Auxin Biosynthesis in *Pisum sativum*: A Physico-Chemical Perspective

By

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DECLARATION

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ABSTRACT

There are a number of putative tryptophan-dependent pathways leading to the bioactive auxin, indole-3-acetic acid (IAA) in plants, although none of these is fully characterised in terms of the isolation of genes for enzymatic reactions, and identification and quantification of precursor compounds. Herein, the tryptamine pathway (tryptophan – tryptamine - indole-3-acetaldehyde - IAA), the indole-3-pyruvic acid pathway (tryptophan - indole-3-pyruvic acid- IAA) and the indoleacetamide pathway (tryptophan – indoleacetamide - IAA) to IAA are examined in *Pisum sativum* (pea) using a compound-based approach, detecting potential precursors by gas chromatography/tandem-mass spectrometry (GC/MS/MS) and ultra performance liquid chromatography/mass spectrometry (UPLC/MS), in order to complement the genetic findings of others. Synthesised deuterated forms of the intermediates involved have been used to quantify the endogenous compounds, and to investigate their metabolic fates. Methods for the isolation and quantification of the following compounds were devised and tested. Tryptophan, tryptamine, indole-3-acetaldehyde (IAAld), indoleacetamide, indole-3-ethanol (IEt) and IAA were detected as endogenous constituents, whereas indole-3-acetaldoxime and one of its products, indole-3-acetonitrile, were not detected. Metabolism experiments indicated that the tryptamine pathway to IAA in pea roots proceeds in the sequence tryptophan – tryptamine – IAAld – IAA, with IEt as a side-branch product of IAAld. The endogenous levels of IAA precursors, along with their abundance in tissue types, of a low-auxin mutant (*bushy*) have also been studied in order to characterise the mutation, and to assess the involvement of different IAA biosynthesis pathways. The effects of wounding, a synthetic auxin and an inhibitor of auxin action on the regulation of the biosynthesis pathways have also been studied. It appears that the tryptamine pathway is operative in pea roots; however, it cannot be ruled out that other tryptophan-dependent pathways to IAA are also operative, nor can we rule out the possibility of a tryptophan-independent pathway operating in these organs.

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ABBREVIATIONS

IAA – indole-3-actetic acid

IAAld – indole-3-acetaldehyde

IPyA – indole-3-pyruvic acid

IAM – indole-3-acetamide

IAN – indole-3-acetonitrile

IAOx – indole-3-acetaldoxime

IBA – indole-3-byturic acid

IEt – indole-3-ethanol

GC – gas chromatography

MS – mass spectrometry

SIM – selected ion monitoring

UPLC – ultra performance liquid chromatography

HP/LC – high performance liquid chromatography

BHT – butylated hydroxytoluene

PCIB – 2-(*p*-chloro-phenoxy)-isobutyric acid

NAA – 1-naphthaleneacetic acid

dH_2O – distilled (MilliQ) water

BSTFA – N,O-bis(trimethylsilyl)trifluoroacetamide

TMCS – trimethylchlorosilane

2,4-D – 2,4-dichlorophenoxyacetic acid

PFB – pentafluorobenzylhydroxylamine

CHAPTER 1

An Introduction to Indole-3-Acetic Acid (IAA) Biosynthesis

1.1. The IAA pool: A result of production, conjugation, transport and degradation

Auxin is a key plant growth hormone, involved in developmental processes as diverse as stem elongation, gravitropism, branching and embryogenesis (Davies, 2004; Liu et al., 1993; Friml et al., 2002; Mattsson et al., 1999). Depending on the concentration and tissue type, auxin either stimulates or inhibits cell elongation (Thimann, 1938). Alterations of auxin levels in plants are triggered by both environmental and developmental cues. For example, gravitropism leads to increased auxin content in the lower side of horizontally placed organs: in shoots, this enhances growth and these organs bend upward, whereas in roots, the high auxin level inhibits elongation, and the root grows downwards (Whippo and Hangarter, 2006). Other environmental effects are also thought to be mediated by auxin levels, for example shade avoidance, where shade-induced changes in light quality trigger local auxin biosynthesis, accelerating stem elongation to reach light (Tao et al., 2008; Benková et al., 2003). Auxin is also involved with the formation of the apical hook in germinating seedlings – a structure that protects the apical meristem – with auxin maxima located under the hook (Friml et al., 2002; Vanneste and Friml, 2009).

The endogenous auxins that have been isolated in plants include indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), phenylacetic acid, and 4-chloro-IAA (Simon and Petrášek, 2011). The latter is a known constituent of certain legume seeds (Katayama et al., 1988). The main auxin in higher plants, IAA, is thought to be required for plant viability, and strongly IAA-deficient mutants are scarce (Normanly et al., 1995; Zhao, 2010), with auxin autotrophs possibly being lethal (Normanly et al., 2004). In any given tissue, the endogenous level of IAA is a consequence of local auxin production, conjugation, intercellular transport and degradation of the hormone (Normanly, 2010; Cheng et al., 2006; Tanaka et al., 2006). The latter three aspects are relatively well understood.

An important aspect of auxin action is the differential distribution of auxin between cells of a tissue, (auxin gradients) and auxin maxima (the accumulation of auxin within a single cell or

small groups of cells; Vanneste and Friml, 2009). Studies have revealed that auxin accumulation in any given cell is capable of modifying developmental programs, in addition to effects on cell elongation. The differential distribution of auxin can signal developmental changes in its own right (Dubrovsky et al., 2008; Reinhardt et al., 2003). Reinhardt et al., for example, found that proteins involved in auxin transport regulate phyllotaxis in Arabidopsis. Again, root hair development relies on auxin gradients, with chemical or genetic disruptions to these gradients dramatically affecting the development of the organs (Aida et al. 2004; Galinha et al. 2007; Overvoorde et al., 2011).

After its synthesis, two pathways are used to translocate auxin: one is the source-to-sink transport of auxin in the phloem from the biosynthesis point to other areas (non-polar auxin distribution), and the other is a slower distribution in a cell-to-cell manner via specific influx (*AUX1*) or efflux (*PIN1*) carrier proteins (polar auxin movement; Tanaka et al., 2006; Vanneste and Friml, 2009; Petrášek and Friml 2009; Schrader et al., 2003). Via either of these routes, IAA is transported to elongating and developing tissues. Thus, the plant regulates its IAA level in any given tissue by both transport into the area, and by local IAA biosynthesis.

Auxin is produced primarily in the shoot apex and young leaves (Ljung et al., 2001a), but evidence has been presented that older leaves of pea also produce IAA (Jager et al., 2007). Other studies have also demonstrated that auxin is synthesized in the roots, and that this root-synthesized auxin contributes to the gradients and maxima required for root development (Ljung et al. 2005; Ikeda et al. 2009; Petersson et al. 2009; Overvoorde et al., 2011).

In order to fully understand the importance and relevance of auxin as a hormone, it is necessary to understand its formation, and the regulation of that process (Lau et al., 2008). This chapter aims to outline the research that has been carried out in the field of IAA biosynthesis in higher plants, and to highlight the gaps in our understanding of the processes involved.

1.1.1. IAA biosynthesis and regulation

Despite decades of research, the routes of IAA synthesis are still the source of much debate (Bartel et al., 2001; Lehmann et al., 2010; Zhao, 2010). The topic has been extensively studied, although, the conclusions drawn have been diverse (for a review, see Bartel et al.,

2001). Indeed, the more IAA biosynthesis is studied, the more the suggested pathways appear to diversify. There are not only often species-related differences in the pathway used to produce the hormone, but also redundancies occurring in a single species (with more than one pathway operating), or multiple genes for single steps in a pathway (Zhao, 2010). Plant species as diverse as rice, tomato, tobacco, and *Arabidopsis* have been studied, and gene families are generally responsible for each biosynthesis pathway step, making the production of IAA biosynthesis mutants very difficult (Zhao, 2010). Furthermore, the pathway used by a particular plant species can vary depending on its environmental conditions, developmental stage, and tissue type (Ljung et al., 2005; Zhao, 2010).

It is probable that the level of IAA in any one tissue type of any one species at any given time is under strict regulatory control. It has been suggested previously that a negative feedback system is in operation to regulate IAA biosynthesis in *Arabidopsis*, where an initial increase of IAA level after treatment with naphthylphthalamic acid (an auxin transport inhibitor) induced an IAA feedback inhibition of IAA synthesis (Ljung et al., 2001a). Conversely, Strader et al. (2011) suggest that a positive feedback system occurs in *Arabidopsis*, with IAA promoting the conversion of indole-3-butyric acid (IBA) to IAA. This is in agreement with the finding that in carrot cell cultures, synthetic auxin application stimulates an increase in the level of IAA (Michalczuk et al., 1992). However, there are few reports documenting the response of IAA biosynthesis pathway intermediates to exogenous auxin application.

Wounding is a model biological system in which to study the regulation of IAA biosynthesis. Sztein et al. (2002) suggest that bean plants respond to wounding by an increase in free IAA levels. Similarly, an IAA pulse was noted in *Arabidopsis* leaves after wounding (Ljung et al., 2001a). However, again, the response that IAA biosynthesis intermediates illicit after wounding has been overlooked.

There is quite a dramatic contradiction apparent in the research of IAA biosynthesis: there is evidence that wounding plants does have an effect on the IAA levels (and presumably its synthesis), and yet many of the studies on IAA biosynthesis have been conducted using parts of plants rather than whole plants, completely overlooking this response. To overcome this issue, whole plants were used throughout this research.

1.2. The production of IAA: The tryptophan-independent pathway

The tryptophan-independent pathway to IAA is suggested to branch off from indole-3-glycerol phosphate or indole, bypassing tryptophan to form IAA (Normanly et al., 1993; Ouyang et al., 2000; shown in Figure 1.1). It is thought that the pathways proceeding through tryptophan, and that bypassing it, may operate in plants at different developmental stages (Michalczuk et al., 1992; Tam and Normanly, 1997), perhaps with the tryptophan-independent pathway as the homeostatic pathway, and the tryptophan-dependent pathway as a response to wounding or stress when high IAA levels are required (Sztein et al., 2002; reviewed in Normanly, 2010). The initial evidence for a pathway bypassing tryptophan came with the discovery of the *orp* mutant in maize, a tryptophan auxotroph – which appeared to have higher levels of IAA (Wright et al., 1991). However, no intermediates have been determined within the tryptophan-independent pathway, despite recent evidence suggesting its involvement in tomato (Ehlert et al., 2008). This thesis will therefore mainly be focussed on the tryptophan-dependent pathways to IAA.

1.3. The production of IAA: The tryptophan-dependent pathways

There is good evidence that the amino acid tryptophan is an early IAA precursor (Normanly et al., 1995), but for any given species it is not clear which downstream pathways to IAA follow. Most evidence for the tryptophan-dependent pathways is based on identification of mutants with altered hormone levels, isolation of putative intermediates, and enzymes capable of catalysing the steps involved, or more recently, genes responsible for encoding these enzymes (Lehmann et al., 2010). Based on these studies, the possible tryptophan-dependent pathways in higher plants are the indole-3-pyruvic acid (IPyA) pathway (Stepanova et al., 2008; Tao et al., 2008), the indole-3-acetaldoxime (IAOx) pathway (Bartel et al., 2001), the indole-3-acetamide (IAM) pathway (Pollmann et al., 2009), and the tryptamine pathway (Zhao et al., 2001), on the basis of the first metabolite of tryptophan (as shown in Figure 1.1). Tryptophan is a logical choice for an IAA precursor because it is ubiquitous as an amino acid, and shares a chemical similarity to IAA (Gibson et al., 1972b). Recent evidence also suggests that indole-3-butyric acid (IBA) be a precursor of IAA. It is unclear whether IBA is a biosynthesis intermediate or a storage form for IAA; however, the IAA derived from IBA is reported to play a role in numerous developmental processes in *Arabidopsis* seedlings (Ludwig-Müller, 2000; Strader and Bartel, 2008; Strader et al., 2010; 2011). It is unclear whether IBA is produced independently of tryptophan, or derived from a tryptophan-dependent pathway, or through IAA itself (Ludwig-Müller, 2000).

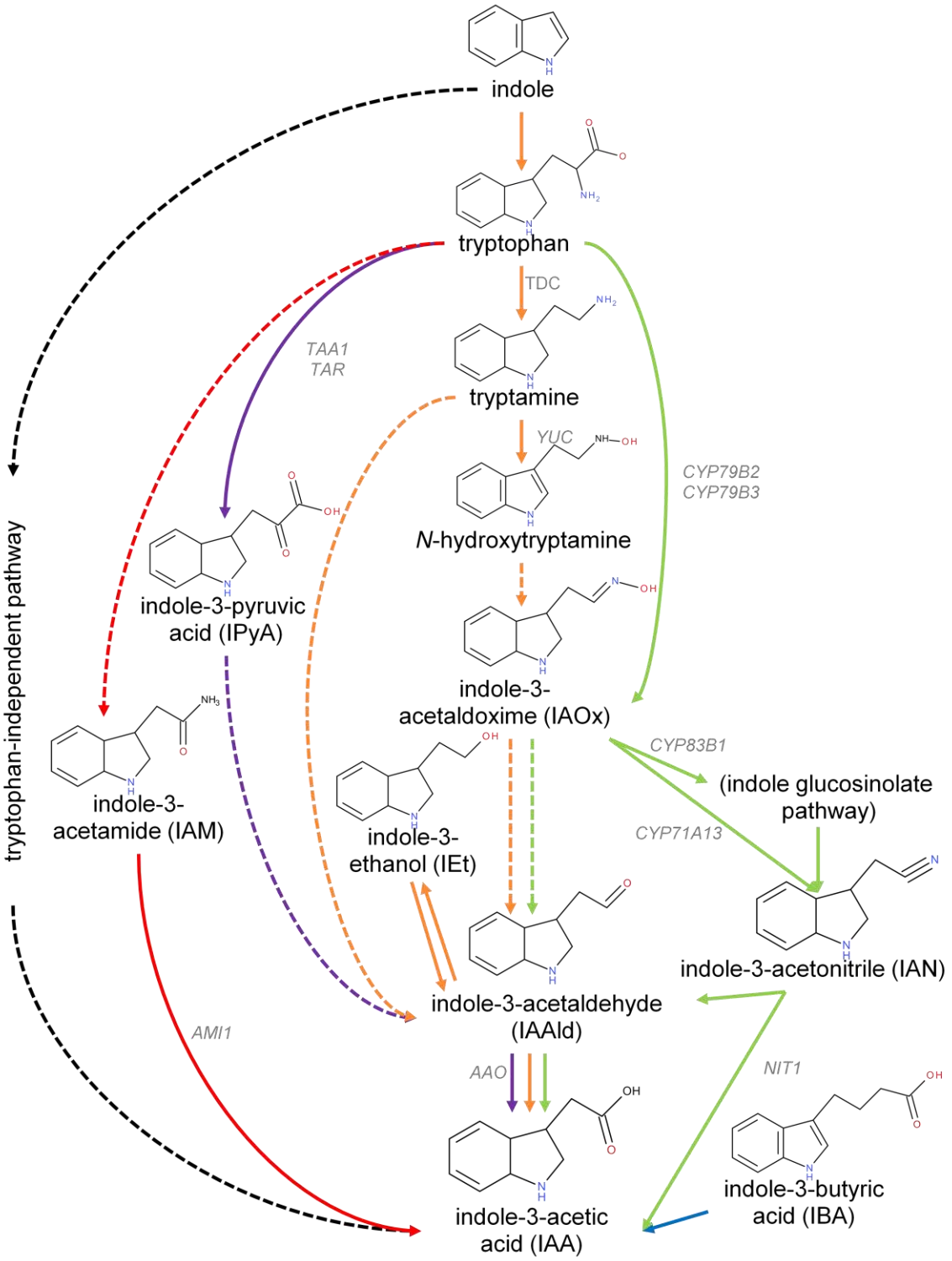

Figure 1.1. The putative tryptamine (orange), IAOx (green), IAM (red), IPyA (purple), and tryptophan-independent (black) biosynthetic pathways to IAA. Shown in blue is the suggested conversion of IBA to IAA. The IAOx pathway (green) is restricted to *Arabidopsis*, and other indole-glucosinolate producing species (Sugawara et al., 2009). Shown in grey are the genes that have been characterised for the steps involved. The dashed lines indicate steps for which no genes have been isolated. Adapted from Lehmann et al. (2010).

Metabolism studies involving the incubation of plant tissue with labelled tryptophan have indicated a transfer of label to IAA on a number of occasions. These include in the sterile root extracts of *Phaseolus coccineus* (Mitchell and Davies, 1972) and *Phaseolus aureus* (Gordon, 1958), cell-free pea extracts (Moore and Shaner, 1967; Erdmann and Schiewer, 1971), *Nicotiana* petiole slices (Liu et al., 1978), and sterile oat coleoptile segments (Heerkloss and Libbert, 1976). However, these studies used out-dated techniques such as thin layer-, or paper chromatography. Techniques for successfully analysing IAA and its precursors have been dramatically improved with the development of precise quantitative methods, namely gas chromatography/mass spectrometry (GC/MS) and liquid chromatography-tandem mass spectrometry (LC/MS-MS). More recently, the conversion of tryptophan to IAA has been shown by the definitive technique of GC/MS in maize endosperm and coleoptile segments (Rakoslavskaya and Bandurski, 1994; Koshiba et al., 1995; Östin et al., 1999; Glawischnig et al., 2000), along with *Arabidopsis* root segments (Müller et al., 1998), tomato shoot segments (Cooney and Nonhebel, 1991; Nonhebel et al., 1993) and sterile germinating bean axes (Bialek et al., 1992). The following sections will briefly introduce evidence for the IPyA, IAOx, IAM and tryptamine pathways to IAA, outlining gaps in the research.

1.3.1. The indole-3-pyruvic acid pathway

The IPyA pathway (reviewed in Chapter 5; proceeding from tryptophan to IPyA to indole-3-acetaldehyde [IAAld] to IAA; Figure 1.1) has been difficult to study because IPyA is unstable in solution during extraction procedures and chromatography (Gibson et al., 1972b; Tam and Normanly, 1998). Recently, Tao et al. (2008) and Stepanova et al. (2008) isolated an *Arabidopsis* tryptophan aminotransferase gene (*TAA1*) that encodes an enzyme responsible for converting tryptophan to IPyA (as confirmed by LC/MS-MS). The *taa1* mutant contains 60% less free IAA, and a reduced IAA synthesis rate in shade conditions (Tao et al., 2008). Interestingly, over-expression of the gene did not cause an IAA overproduction phenotype, suggesting a possible feedback system, or redundancies in this step in the pathway (Stepanova et al., 2008). IPyA has been isolated in *Arabidopsis* seedlings (Tam and Normanly, 1998), tomato (Cooney and Nonhebel, 1989; Cooney and Nonhebel, 1991) and pea root nodules (Badenoch-Jones et al., 1984) by GC/MS. In addition, incubation of tomato and *Avena* plants with [^{14}C]tryptophan showed an accumulation of labelled IPyA, consistent with a precursor role (although this used out-dated techniques; Schneider et al., 1970; Gibson et al., 1972b). The step from IPyA to IAAld has not been fully characterised in plants (Nonhebel et al., 1993; Normanly, 2010). There have been very few studies using up-to-date

techniques (such as GC/MS and LC/MS) to demonstrate the presence of IPyA in higher plants, and its conversion to IAA *in vivo*. Despite this, the recent findings of Tao et al (2008) and Stepanova et al. (2008) have brought the IPyA pathway back into the IAA biosynthesis spotlight (Friml and Sauer, 2008).

1.3.2. The indole-3-acetaldoxime pathway

The IAOx pathway (proceeding from tryptophan [or N-hydroxytryptamine] to IAOx to indole-3-acetonitrile [IAN] and/or IAAld to IAA; Figure 1.1; reviewed in Chapter 4) is thought to be important primarily in the order Capparales (which includes *Arabidopsis*), members of which accumulate indole glucosinolates that possibly act as plant defence compounds (Mikkelsen et al., 2000; reviewed in Normanly, 2010). IAOx is a branchpoint to the indole-3-glucosinolate and IAA pathways (Zhao et al., 2002), with two Arabidopsis P450 monooxygenases, CYP79B2 and CYP79B3 oxidising tryptophan to IAOx (Mikkelsen et al., 2000). However, the compound IAOx itself is also thought to be involved with the tryptamine pathway via *N*-hydroxytryptamine, although there is little evidence for this conversion, or the presence of the latter compound in plants at all (Tivendale et al., 2010). The main evidence for the IAOx pathway comes with studies of cytochrome P450 genes, for example *Arabidopsis SUR2*, encodes a protein responsible for oxidising IAOx in the biosynthesis of indole-glucosinolates (CYP83B1; Barlier et al., 2000). The loss-of-function *sur2* mutant exhibits an elevated IAA phenotype and an accumulation of IAAld, presumably because of an accumulation of IAOx redirected into the IAA pathway instead of the indole-glucosinolate pathway (Barlier et al., 2000; Smolen and Bender, 2002). Therefore, the IAOx pathway may form IAA as a secondary metabolite of the indole-glucosinolates (at least in part), rather than lead to IAA directly, suggesting that the pathway operates only in indole-glucosinolate producing species (Barlier et al., 2000; Bak et al., 2001; Mikkelsen et al., 2004). This is supported by the finding that IAOx is not an endogenous constituent of rice and maize (Sugawara et al., 2009). It is possible that IAOx may also be an intermediate in a pathway to IAA via IAN, however there is little evidence for the conversion of IAOx to IAN, and Arabidopsis IAN may also be produced as a down-stream product of the glucosinolates (Mikkelsen et al., 2000; Normanly et al., 1993). Despite the profusion of research conducted on the IAOx pathway, the evidence for its involvement in IAA synthesis is restricted mainly to *Arabidopsis*, with confirmation of its absence in rice and maize (two monocotyledonous species). In order to confirm that the pathway is restricted to certain dicotyledonous families,

the possible involvement of IAOx as an IAA biosynthesis intermediate in the legume *Pisum sativum* L. (pea) is examined in this thesis.

1.3.3. The indole-3-acetamide pathway

The IAM pathway to IAA (tryptophan to IAM to IAA; Figure 1.1; reviewed in Chapter 6) was previously thought to be active only in bacteria. It is probably the most fully characterised pathway, due to research on these organisms. Recent studies indicate that the pathway may also operate in plants (reviewed in Lehmann et al., 2010). The two-step pathway involves a monooxygenase to convert tryptophan to IAM, and subsequently a hydrolase to convert IAM to IAA, with genes for these steps having been cloned and characterised in many different bacterial genera (Spaepen et al., 2007). Despite early reports of the compound's presence in higher species, due to the possibility of bacterial contamination in earlier studies, only recent evidence will be discussed. The compound has been isolated by GC/- or LC/MS in aseptically grown *Arabidopsis* seedlings (Pollmann et al., 2002), and in maize, rice and tobacco tissues (Sugawara et al., 2009). Pollman et al. (2009) reported the conversion of isotope-labelled tryptophan to IAM, and IAM to IAA. The same group have also demonstrated that the conversion of labelled tryptophan to labelled IAA in protein extracts of *Arabidopsis* was significantly lowered on addition of unlabelled IAM. Furthermore, the cloning and characterization of *Arabidopsis* IAM hydrolase (*AMI1*; Pollmann et al., 2003; 2006b), and an orthologue in tobacco (*NtAMI1*; Nemoto et al., 2009) provided further evidence of the pathway's involvement in higher plant species. However, before the pathway can be accepted as a route to IAA biosynthesis in higher plant species, a wider array of species must be examined. Few dicotyledonous species have been studied recently (namely *Arabidopsis* and tobacco), and in order to examine whether the pathway is widespread, again, the legume *Pisum sativum* L. (pea) is studied in this thesis.

1.3.4. The tryptamine pathway

During the 2000s, there was a renewed focus on the tryptamine pathway (Figure 1.1; reviewed in Chapter 4) as the dominant route to IAA biosynthesis. Zhao et al. (2001) discovered *Arabidopsis YUCCA*, a gene encoding a flavin-containing mono-oxygenase that was reported to convert tryptamine to *N*-hydroxytryptamine, a putative rate-limiting tryptamine pathway step (see also Kim et al., 2007). Orthologous genes have also been isolated in rice (Yamamoto et al., 2007), *Medicago* (Cheng et al., 2006), *Petunia* (*FLOOZY*; Tobeña-Santamaria et al., 2006), tomato (*ToFZY*; Expósito-Rodríguez et al., 2007) and maize

(*THREAD*; Marsch-Martinez et al., 2002; *spi1*; Gallavotti et al., 2008). Over-expression of *YUCCA* results in IAA accumulation in *Arabidopsis* (Zhao et al., 2001), and in functional assay conditions, *YUCCA* was reported to convert tryptamine to *N*-hydroxytryptamine (Zhao et al., 2001; LeClere et al., 2010). However, recent studies challenged the identification of the *N*-hydroxytryptamine product in these papers (Tivendale et al., 2010).

The initial conversion of tryptophan to tryptamine by tryptophan decarboxylase (*TDC*) has been studied to a limited extent, with the corresponding genes and enzymes reported in *Catharanthus roseus* (De Luca et al., 1989), *Camptotheca acuminate* (Lopez-Meyer and Nessler, 1997), *Ophiorrhiza pumila* (Yamazaki et al., 2003) and *Oryza sativa* (Kang et al., 2007), but not in *Arabidopsis* or pea. Interestingly, the transcription of tryptophan decarboxylase in *Camptotheca acuminate* appears to increase in response to stress, possibly due to its function in camptothecin production – which relates to plant defence (Lopez-Meyer and Nessler, 1997).

Tryptamine has been isolated by GC/MS in tomato (Cooney and Nonhebel, 1991), rice (Ishihara et al., 2008) and *Arabidopsis* (Sugawara et al., 2009). On the basis of out-dated techniques, it appears that [^{14}C]- forms of tryptamine are converted to IAA in tobacco apices (Phelps and Sequeira, 1967), and to the putative downstream intermediates IAAld and IEt, as well as IAA, in tomato and barley (Schneider et al., 1970; Gibson et al., 1972b). The conversion of [^{14}C]- labelled tryptophan to tryptamine is a result noted in *Nicotiana* callus (Liu et al., 1978) and terminal buds (Phelps and Sequeira, 1967), and tomato and barley shoots (Schneider et al., 1970). However, these studies were not carried out using the definitive techniques of mass spectrometry. More recently, a study using LC/MS found that [^{15}N]tryptamine supplied to *Arabidopsis* was converted to IAA, but not to IAOx (Sugawara et al., 2009), suggesting that IAOx may not be a tryptamine pathway constituent. It is important to confirm this result in another dicot, pea, and to determine through which intermediates tryptamine is converted in order to produce IAA.

1.4. The hazards of studying IAA biosynthesis

It is difficult to study a biosynthetic pathway that appears to be functionally redundant. Compounding this, with few loss-of-function mutants, thorough examination of the regulatory roles of intermediate steps in the pathway becomes problematic. Determining the existence of each possible pathway is difficult without being able to specifically inhibit steps

in those pathways. Furthermore, the hypothesised pathways may be interlinked, with the putative cross-talk between them causing problems for the study of each one.

There is a serious shortfall in the research involving the tryptophan-dependent pathways in that few studies have yet been carried out using GC/MS or LC/MS to examine putative IAA precursors and to determine the products of labelled intermediates (reviewed in Normanly et al., 1995). This is probably because of the lack of isotope-labelled putative precursors available commercially. In order to establish the sequence of reactions in the IAA biosynthesis pathway, the compounds involved must be found endogenously, they should be converted *in vivo* to IAA, metabolised from tryptophan, and to appropriate compounds in a pathway cascade to IAA (Woodward and Bartel, 2005). Furthermore, an evaluation of the isotope enrichment of putative pathway intermediates must be carried out. The IAA biosynthesis pathways have not previously been studied in many of these aspects, or in whole plant systems using GC/MS and isotope labelled compounds. In this aspect, the current research is unique.

1.5. The aims of this thesis

The aim of this thesis is to discover more about IAA biosynthesis in the vegetative tissues of *Pisum sativum*, by identifying precursors to the hormone and conducting metabolism studies. The thesis deals most specifically with the putative tryptamine, IAOx and IAM pathways to IAA. In order to determine the presence of previously-documented IAA biosynthesis pathways, and their specific routes to IAA, the model species *Pisum sativum* (pea) was investigated. Pea was used throughout this study in order to examine a dicotyledonous species other than *Arabidopsis*, on which the majority of recent research has been carried out. *Arabidopsis* and pea are quite different in terms of growth habit, and pea is an important Australian crop species.

Another advantage of the current research is that no previous single publication has examined all pathways together in one species at one time using sophisticated physicochemical techniques throughout. The major questions asked here were: 1) Can the pathways operate in peas? 2) If they can operate, through which intermediates do they proceed? and 3) Are the pathways regulated?

The thesis aims to examine aspects of the regulation of IAA content. In particular, the effects of wounding and auxin application on the levels of key pathway intermediates, and of IAA itself, will be examined. The response of a plant to wounding represents a model biological system in which to examine the mechanisms that play a role in IAA regulation. Likewise, examining the response of pea plants to exogenous IAA application may also uncover feedback regulation mechanisms at work *in vivo*. Moreover, a pea mutant exhibiting lower free IAA content, *bushy* (Symons et al., 2002), is further characterised with regard to levels of IAA precursors, which may shed further light on the principal biosynthesis pathways involved in pea: a unique tool to study IAA biosynthesis.

CHAPTER 2

General Materials and Methods

Parts of this chapter have been published in:

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2.1. Plant material and glasshouse conditions

Unless otherwise stated, pea plants (*Pisum sativum* L.) of line 107 were used throughout the metabolism and quantification experiments, obtained from the collection held at the School of Plant Science, University of Tasmania (as shown in Figure 2.1). Line 107 (Torsdag) is wild-type with respect to internode length genes. Also used were seeds of the heterozygous HL297, *BSHbsh*. These seeds segregated in a 1:2:1 ratio of WT, heterozygote and *bushy* mutant plants (the *bushy* mutant is characterised by Symons et al., 1999; see Chapter 7). In studies involving quantification, seed testae were nicked prior to planting with a sterile razor blade to promote uniform germination, and all pots were sterilised with 70% ethanol prior to use. Pea plants were grown, two per pot, in 14 cm slim line pots, using a 1:1 mixture of vermiculite and dolerite chips topped with a 2-3 cm layer of sterilized peat-sand potting mixture. Nutrient solution was applied once per week (Aquasol, Hortico Ltd, Melbourne, Australia), and plants were watered four times a week after first leaf expansion. All plants were grown in a heated glasshouse with day temperatures of 20-25°C, and night temperatures of ~16°C. Day length was extended before dawn and after dusk to 18-hours, with light from a mixed fluorescent (Thorn, 40 W white tubes)-incandescent (Mazda, 100 W pearl globes) source, providing 35 μmol m^{-2} s^{-1} at pot top, as described previously (Reid and Potts, 1986; Beveridge and Murfet, 1996).

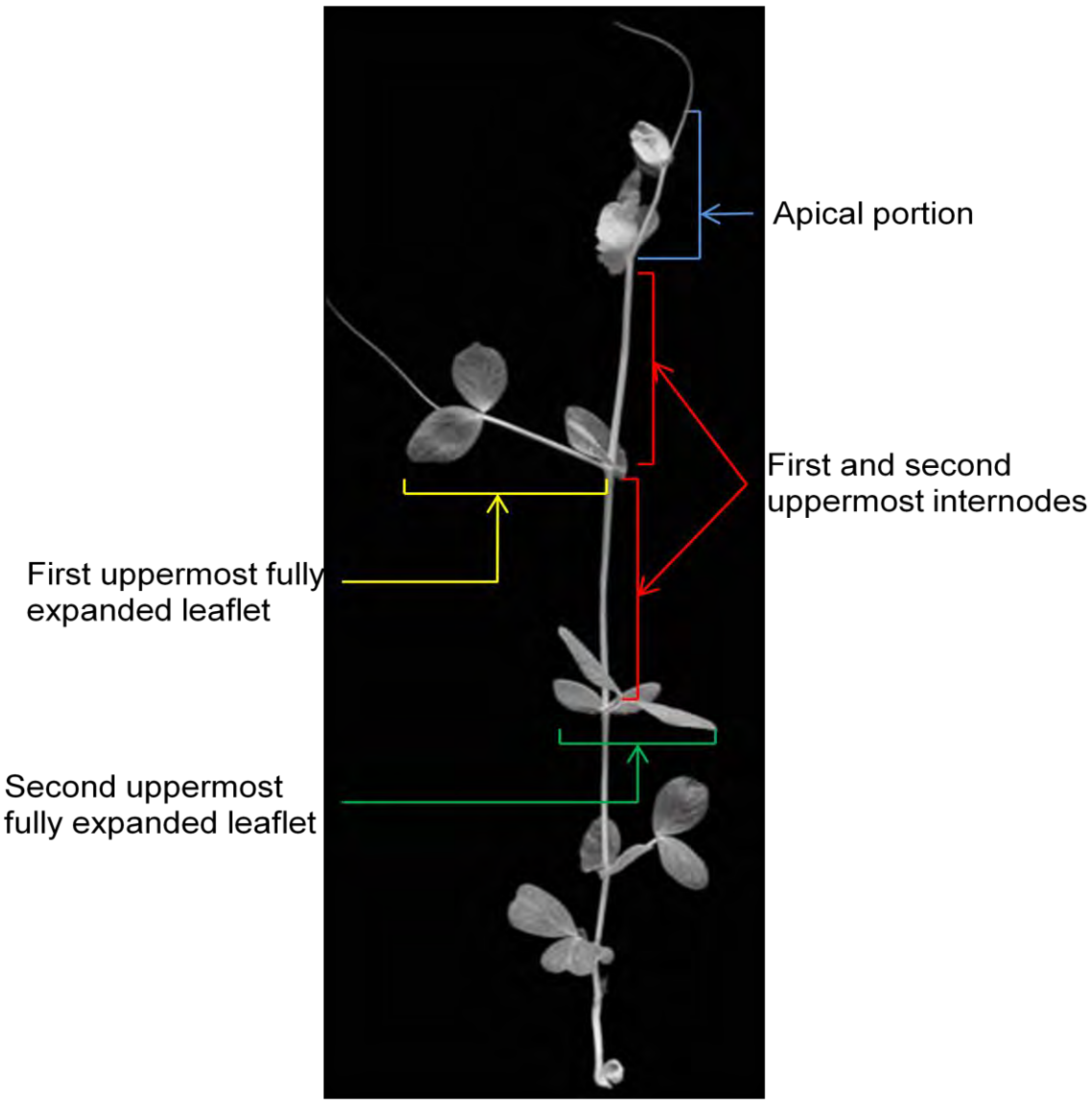

Figure 2.1. A three-week-old pea plant of line 107. This shows the separation of pea shoot tissue into leaf tissue (both the first (yellow) and second (green) uppermost fully expanded leaflets and stipules), internode tissue (first and second uppermost internodes; red), and apical portions (including un-expanded leaflet and apical bud; blue), harvested for hormone analysis.

2.1.1. Harvested material

Three week old pea tissue was used for quantification experiments unless otherwise stated. In experiments where shoot material was separated, internode segments included the uppermost two internodes, leaf material included the two uppermost expanding leaves and stipules, and the apical tissue included the apical bud only (as shown in Figure 2.1). Seven day old root material (excised at the seed) was washed in dH_2O, and blotted before weighing. In other experiments, whole plant material was harvested above the second-uppermost fully expanded leaf, and the entire apical portion used for quantitation (Figure 2.1). In all cases, 3-4 plants were used per replicate.

Once harvested and weighed, the material was placed in beakers, and covered with 80% methanol containing 250 mg L^{-1} butylated hydroxytoluene (BHT; Sigma-Aldrich, St. Louis, MO, USA). This material was placed in a freezer at -20°C for a minimum of one hour. Following this, samples were homogenized and extracted in 80% methanol at 4°C for 24 hours. Samples were then filtered using Whatman No. 1 filter paper, with the particulate matter washed three times with 80% methanol. The filtrate was stored in the freezer at -20°C. For those experiments involving tryptophan quantification, plant material was harvested and extracted in dH_2O containing 250 mg L^{-1} BHT at 4°C and all filtration was carried out in dH_2O. For all quantification studies, a known amount of appropriate internal standard was added to the extracts after filtration. All internal standards apart from [$^2H_5$]tryptophan were added at the level of 10 ng $g(FW)^{-1}$ into raw extracts after filtration: [$^2H_5$]tryptophan was added at 500 ng $g(FW)^{-1}$. In all circumstances, the equivalent aliquot of approx. 1 g FW tissue was then used for hormone/compound quantification.

2.1.2. Studies investigating the metabolism of intermediates: Protocols for aseptic germination and incubation with labelled precursors

For metabolism purposes, pea seeds were sterilized in 70% ethanol for one minute, followed by a sodium hypochlorite solution containing 0.2% available chlorine for five minutes, before being rinsed several times in sterile dH_2O. In a lamina flow hood, seeds were planted (just underneath the medium) in 10 mL of autoclaved revised Murashige and Skoog (1962) medium (Sigma, St. Louis., MO, USA) containing 1.2% agar and 20 g L^{-1} sucrose as additives, in 15 mL Falcon tubes. Seeds were positioned with the radicle pointing down into the agar in order to promote straight root growth. Tube lids were screwed half on to maintain the tube sterility while permitting gas exchange, and then placed in a growth cabinet, in the

dark at 20°C for seven days. The germination rate for this technique was approximately 75%. Seedlings were then transplanted (in a laminar flow hood) into tubes containing sterile dH_2O that had been aerated for one hour. Control plants were placed in dH_2O, whereas treated plants were placed in dH_2O containing the compound of interest. For [$^2H_4$]- and [$^2H_5$]tryptamine the concentration was 10.8 μM, for [$^2H_5$]tryptophan: 8.5 μM, for [$^2H_5$]IAOx: 1 μM and for [$^2H_5$]IAN: 2 μM, with 3 to 4 seedlings placed in each 15 mL tube. All other labelled compound concentrations are outlined in subsequent chapters. Plants were maintained under these conditions, in a lamina flow hood for 24 hours, and then harvested from below the seed – i.e. the roots only. Selected experiments included a harvest of the whole shoot tissue above the seed, separately, to examine possible uptake from the root to the shoot tissue. In some experiments, the H_2O of both control and treated plants was also sampled. The material harvested was then subjected to the same homogenisation and extraction conditions used for quantitation purposes (Chapter 2.1.1). When possible, the amount of label taken up and converted to downstream intermediates was examined using other stable-labelled forms (that is, not [$^2H_5$], generally [$^{13}C_6$]-, [^{14}C]-, or [$^2H_2$]-labelled).

In one experiment, leaflets were applied with [^{14}C]tryptophan. [^{14}C]tryptophan in 80% methanol, or 80% methanol in dH_2O with no added hormone, was applied to the second uppermost fully expanded leaflets of three week old whole pea plants grown as described (Chapter 2.1). [^{14}C]Tryptophan level applied was at 100,000 dpm per plant, with two leaflets of each plant treated. After 24 hours, treated and untreated leaflets were excised at the node, washed in dH_2O, and then subjected to the same homogenisation and extraction conditions used for quantitation purposes (Chapter 2.1.1).

In some metabolism experiments, and all quantification experiments, internal standards were added to quantify metabolites. [$^2H_5$]- and [$^2H_2$]- labelled forms used for metabolism studies and internal standards were synthesized in the School of Chemistry, University of Tasmania, by Dr. Jason Smith and Dr. Peter Molesworth, and [$^2H_4$]tryptamine (α-$^2H_2$:β-$^2H_2$; Medical Isotopes Inc., Pelham, NH) and [$^{13}C_6$]IAA (Cambridge Isotope Laboratories, Andover, MA) were also used. All internal standards apart from [$^2H_5$]tryptophan were added at the level of 10 ng g(FW)$^{-1}$ into raw extracts after filtration: [$^2H_5$]tryptophan was added at 500 ng g(FW)$^{-1}$. The label positions for all compounds are presented in Figure 2.2.

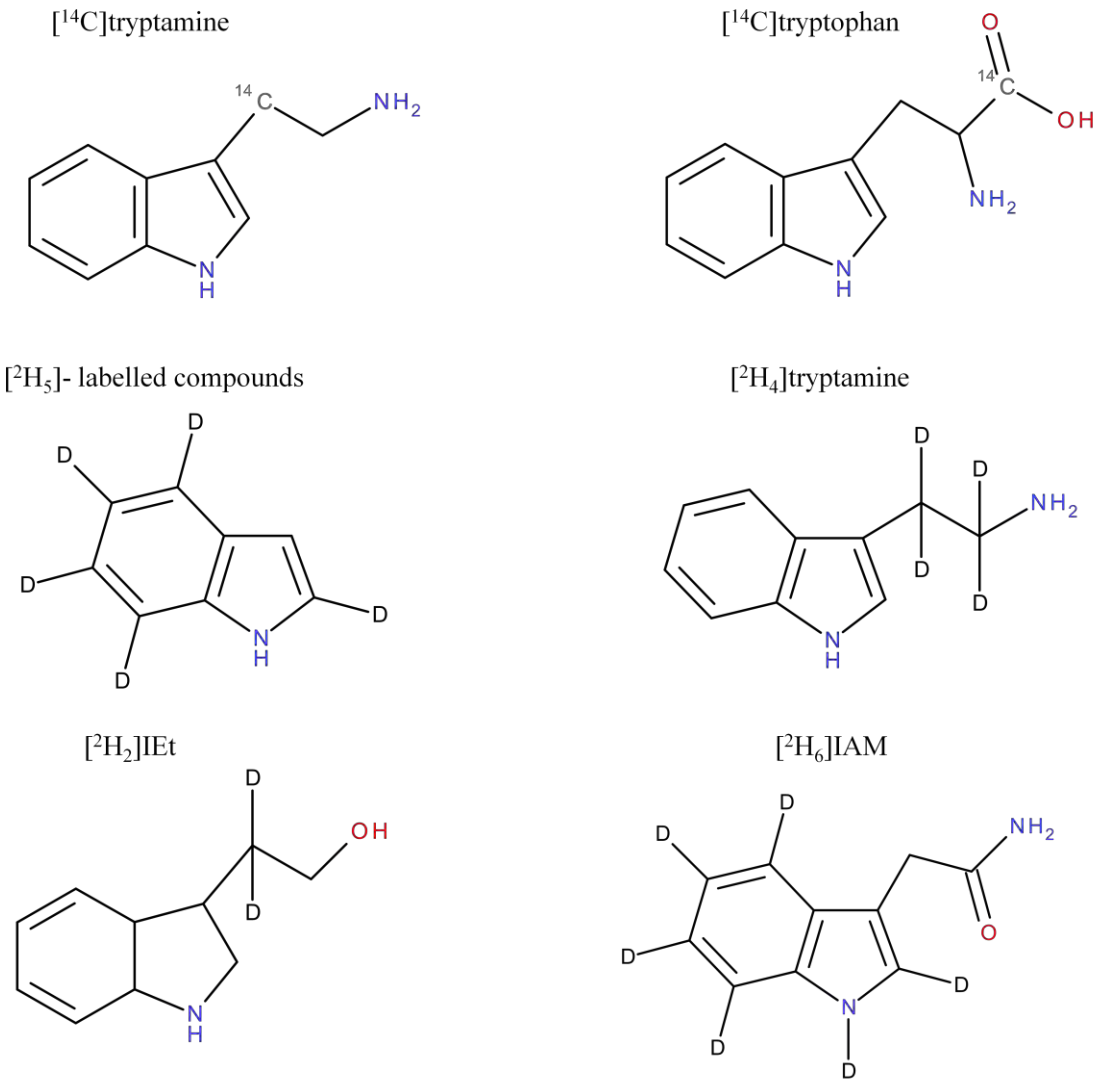

Figure 2.2. The positioning of label on compounds used for GC/MS and UPLC/MS analysis. From left to right (top to bottom): [^{14}C]tryptamine, [^{14}C]tryptophan, [^{2}H$_5$]- labelled compounds (tryptophan, tryptamine, IAOx, IAN), [^{2}H$_4$]tryptamine, [^{2}H$_2$]IEt and [^{2}H$_6$]IAM.

2.2. Preparation of extracts for the isolation and quantification of compounds by GC/MS/MS analysis

For the development of these techniques, see Chapter 3. The sources for unlabelled standard material for all intermediates measured are noted in Chapter 3.

2.2.1. Tryptophan

Aqueous tryptophan-containing extracts were acidified to pH 2 using 1N HCl, then 400 µL penta-fluoro-propanol (Fluka, Steinheim, Germany) and 100 µL pyridine were added, and the samples vortexed for five seconds. After this, 100 µL of ethyl chloroformate (Merck, Hohenbrunn, Germany) was added, and the sample was vortexed for ten seconds, and left to stand for ten minutes (as described previously by Perrine et al., 2004). The sample was then partitioned with 200 µL of chloroform three times, and the organic phase dried under N_2, and taken to complete dryness in a sample concentrator (DYNAVAC condenser coupled with a LABCONCO Centrivap concentrator, Kansas City, Missouri, USA).

Trimethylsilylation was then performed by adding 40 µL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA+1%TMCS; Grace Davidson, Deerfield, IL, USA) containing 1% trimethylchlorosilane (TMCS) to the dry samples with 10 µL pyridine to aid dissolution, and heating to 80°C for 30 minutes. Subsequently, the extracts were dried under N_2 and 15 µL BSTFA (1% TMCS) was added. Samples were then placed in the oven at 80°C for a further 30 minutes. The tryptophan carboxyl group reacts with the pentafluoropropanol to become esterified, the amine group reacts with the ethyl chloroformate to form an ethylformyl derivative, and the indole nitrogen reacts with the BSTFA (1% TMCS), forming a trimethylsilyl derivative. The resulting derivative is N-ethoxycarboxyl trimethylsilyl pentafluoropropyl ester, as shown in Figure 2.3.

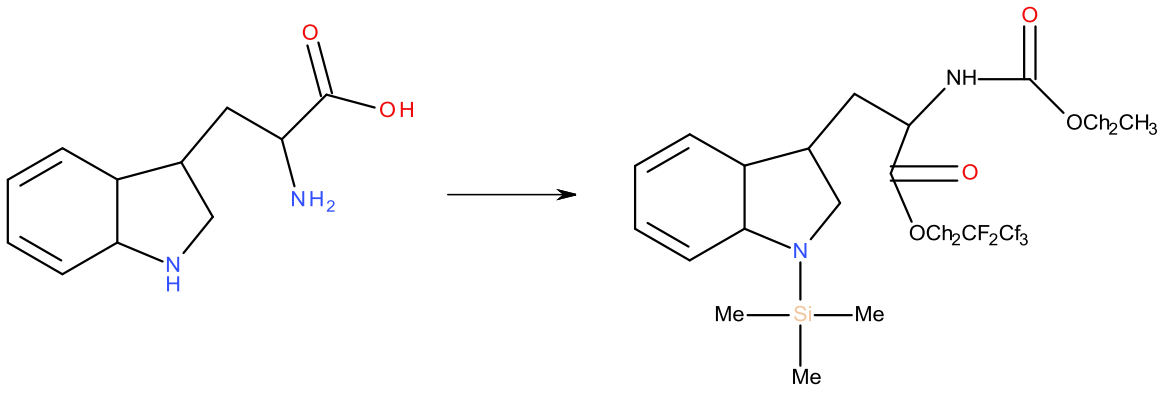

Figure 2.3. The structure of tryptophan before (left) and after derivatisation with pentafluoropropanol, ethyl chloroformate, and BSTFA (1% TMCS), to form the N-ethoxycarboxyl trimethylsilyl pentafluoropropyl ester derivative (right).

2.2.2. Tryptamine

For analysis of tryptamine, extracts in 80% methanol were reduced under vacuum at 30°C to a small volume (1 to 4 mL), then taken up in 2 mL of 2% acetic acid in dH_2O, and partitioned three times with 1 mL chloroform. The organic phase was discarded, and the aqueous phase taken to pH 11, using 1N NaOH, and partitioned with chloroform again. The organic phase was dried under N_2, transferred to a tapered-bottom vial, and taken to complete dryness in a sample concentrator. In some studies, extracts were subjected to High Performance-Liquid Chromatography (HP/LC), as previously described (Jager et al., 2005). The system consisted of a 1525 Binary HPLC Pump, a Rheodyne Manual Injector fitted with a 2 ml sample loading loop, a 2487 Dual Absorbance Detector, and a Bondapak C_{18} 10 μm, 100 mm × 8 mm Radial-Pak cartridge. The solvents used were 1% acetic acid (A) and acetonitrile (1% acetic acid; B), at one mL/min over a gradient of 10-60% B for 20 mins, followed by 60-100% B over the next 10 mins. Tryptamine eluted after 15-16 minutes. After being taken to complete dryness, all samples were acylated using 100 μL of both pentafluoropropionic anhydride (Alltech, Drive State College, PA, USA) and acetonitrile in a 60°C oven for 90 minutes (as described previously by Martínez and Gelpí, 1979; Sobolevsky et al., 2003). The indole nitrogen and side-chain amine groups both react with the pentafluoropropionic anhydride to form a bis-pentafluoropropionyl derivative as shown in Figure 2.4.

2.2.3. Indole-3-pyruvic acid

Extracts for the analysis of IPyA were reduced under vacuum at 30°C to a small volume (1 to 4 mL). Extracts were purified with Sep-Pak C_{18} cartridges (VAC RC 500 mg; Waters, Ireland) pre-conditioned with 100% methanol followed by 0.4% acetic acid in dH_2O. Three 1 mL washes of 0.4% acetic acid were used to load the extract, and 10 mL 100% methanol was used to elute the IPyA into a McCartney vial. To this eluate, one BHT crystal, and pentafluorobenzylhydroxylamine (PFB; Sigma-Aldrich, St. Louis, MO, USA) was added (300μg in 1g fresh weight plant tissue), the vial was flushed with N_2, then capped and heated at 50°C for 90 minutes (as described by Tam and Normanly, 1998). Subsequently, the excess reagent was reacted with acetone (200 μL), then dried under N_2.

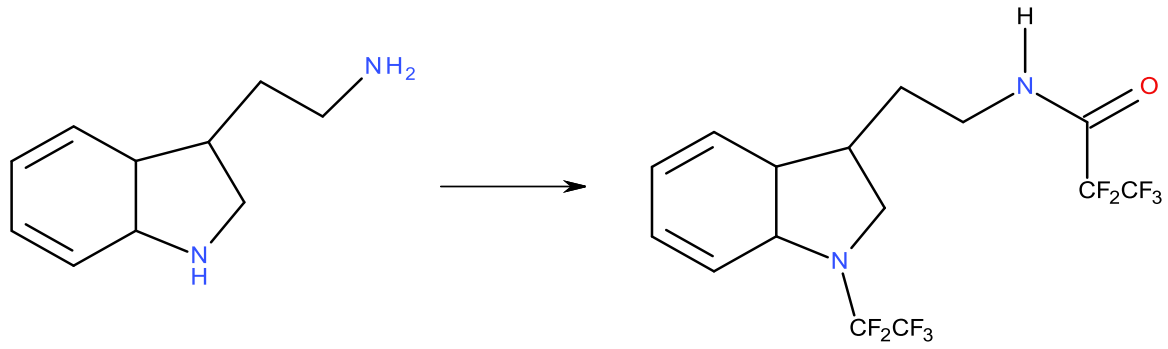

Figure 2.4. The structure of tryptamine before (left) and after derivatisation with pentafluoropropionic anhydride, to form a bis-pentafluoropropionyl derivative (right).

In order to successfully analyse samples by GC/MS/MS, the excess reagent needed to be removed. Therefore, the extract was again passed through a pre-conditioned Sep-Pak C_{18} cartridges as described above, rinsed with 30% methanol, and eluted off the column with 15 mL 100% methanol. This eluate was dried under vacuum, and was then taken up in 20 mL dH_2O, and partitioned three times with 15 mL chloroform. The organic phase was then dried under vacuum, and transferred in methanol to a tapered bottom vial.

Methylation was performed by adding 200 µL of methanol and 750 µL of (Trimethylsilyl)diazomethane solution (2.0 M in hexanes; Sigma-Aldrich, St. Louis, MO, USA), and leaving for 30 minutes. After this, the extract was evaporated to dryness under a N_2 stream in a block heater. Trimethylsilylation was then performed as described in Chapter 2.2.1. The hydroxylamine from the PFB reagent reacts with the ketone group, producing an oxime (pentafluorobenzyl oxime). The diazomethane reacts with the carboxyl group, forming a methyl ester. The BSTFA (1% TMCS) reacts with the indole nitrogen, forming a trimethylsilyl derivative. Therefore, the derivative is a pentafluorobenzyl oxime trimethylsilyl methyl ester as shown in Figure 2.5.

2.2.4. Indole-3-acetaldoxime

Extracts for the analysis of IAOx were reduced to dryness under vacuum at 30°C. Extracts were loaded on to preconditioned (as described in Chapter 2.2.3) Sep-Pak C_{18} cartridges in 3 1 mL washes of 0.4% acetic acid and eluted with 10 mL 50% methanol (0.4% acetic acid). Again, after being taken to complete dryness in a rotary evaporator, the extracts were then fractionated using a reverse-phase C_{18} HP/LC system as described in Chapter 2.2.2 (Waters Associates, Milford, USA). The solvent program was a linear gradient from 20-75% methanol in 0.4% acetic acid over 25 minutes, at a flow rate of 2 mL min^{-1}. Extracts were resuspended in 2 x 1 mL washes of 20% methanol in 0.4% acetic acid, and filtered through a 0.45-micron filter (Supor Acrodisc 25; Pall Corporation, MI, USA) prior to injection. A Frac-100 Fraction Collector (Pharmacia Corporation, NJ, USA) was used to collect fractions at one minute intervals over the gradient period. Fractions corresponding to the retention time of IAOx (usually 14-15 mins) were dried under vacuum at 30°C, transferred in methanol to a GC/MS vial, and subsequently dried under N_2. Trimethylsilylation was then performed as described in Chapter 2.2.1. The BSTFA (1% TMCS) reacts with the indole nitrogen and the hydroxyl group, adding two trimethylsilyl groups that give the derivative shown in Figure 2.6.

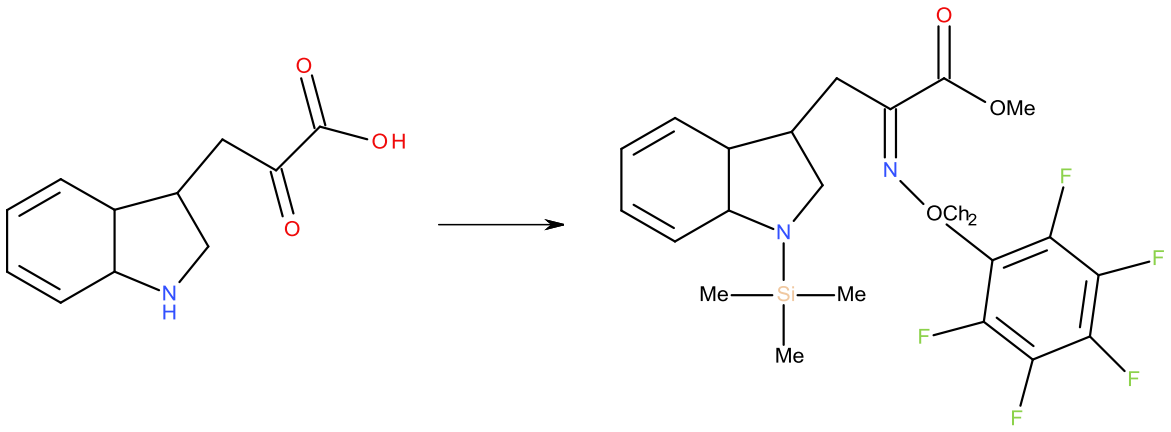

Figure 2.5. The structure of IPyA before (left) and after derivatisation with PFB, diazomethane and BSTFA (1% TMCS), to form a pentafluorobenzyl oxime trimethylsilyl methyl ester derivative (right).

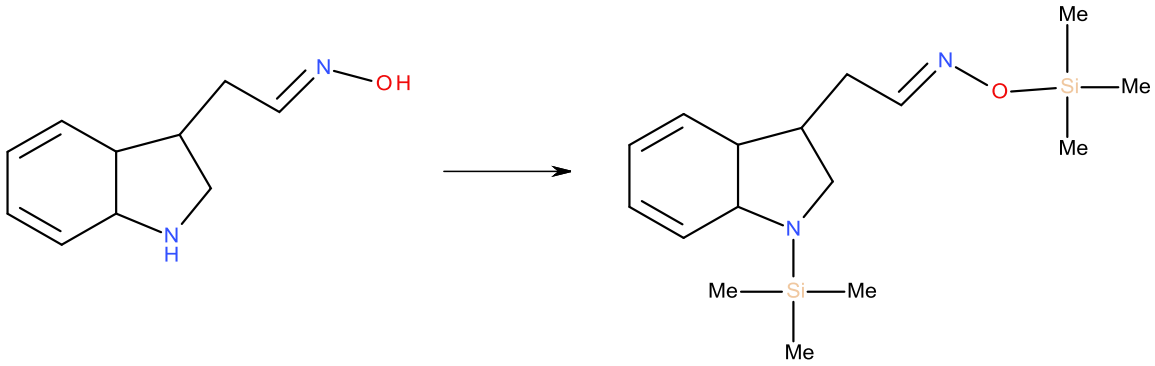

Figure 2.6. The structure of IAOx before (left) and after derivatisation with BSTFA (1% TMCS), to form a bis-trimethylsilyl derivative (right).

2.2.5. Indole-3-acetaldehyde and indole-3-ethanol

Extracts for the quantification of IAAld were stirred on a block heater, with 1g of sodium borohydride per 50 mL extract (Sigma, Dt. Louis, MO, USA), added twice over a nine hour period, to reduce IAAld to IEt (as previously noted by Moore and Shaner, 1968). Subsequently, the extract was dried under vacuum at 30˚C. Extracts for the analysis of IAAld and IEt were taken up in 30 mL of $KHSO_4$ (0.3 N) and dH_2O respectively, and partitioned three times with 10 mL chloroform. The organic phase was then dried under rotary evaporation, transferred to a tapered-bottom vial, and taken to complete dryness in a sample concentrator. Trimethylsilylation was then performed as described in Chapter 2.2.1. The BSTFA (1% TMCS) reacts only with the indole nitrogen in the aldehyde, but also with the hydroxyl group on the side-chain when in the enol form - forming two trimethylsilyl derivatives. The two forms are generally in equilibrium (the derivatives are shown in Figure 2.7). For IEt, The BSTFA (1% TMCS) reacts with the indole nitrogen and the hydroxyl group, adding two trimethylsilyl groups producing the derivative shown in Figure 2.8.

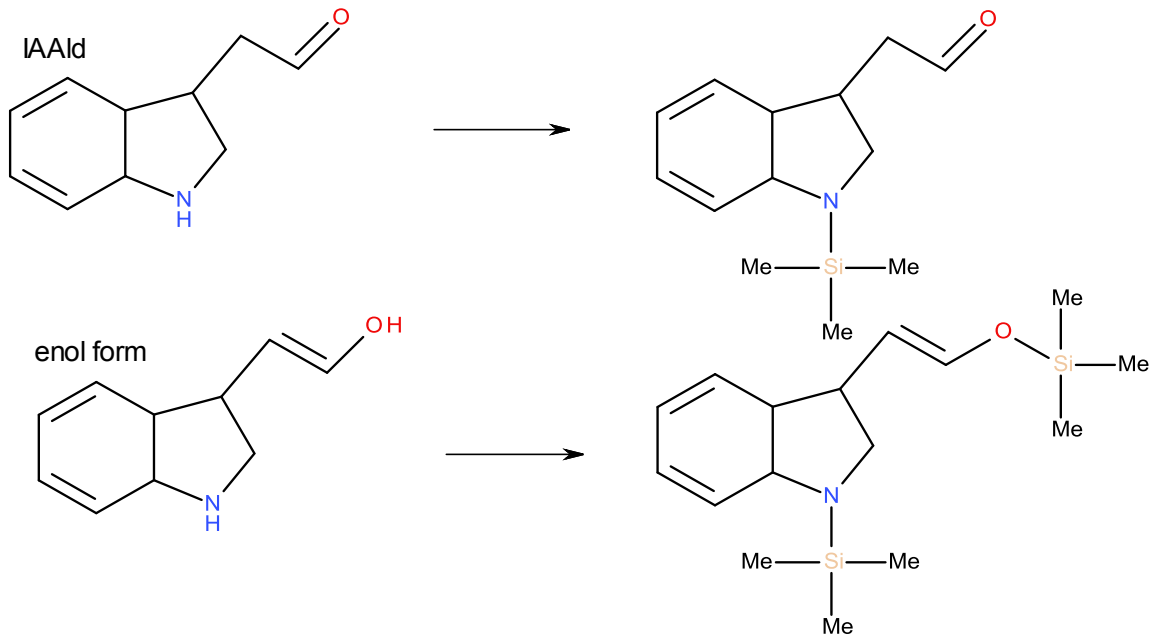

Figure 2.7. The structure of IAAld before (left, top) and after derivatisation with BSTFA (1% TMCS), to form a trimethylsilyl derivative (right, top). In the enol form (bottom left), the BSTFA (1% TMCS) also reacts with the hydroxyl group on the side-chain - forming a bis- trimethylsilyl derivative (bottom right). The two forms are generally in equilibrium.

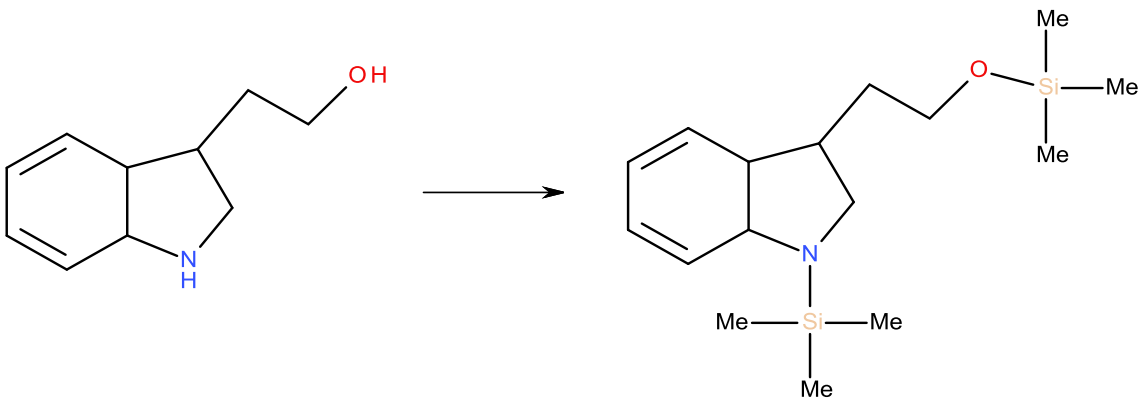

Figure 2.8. The structure of IEt before (left) and after derivatisation with BSTFA (1% TMCS), to form a bis-trimethylsilyl derivative (right).

2.2.6. Indole-3-acetonitrile and IAA

Extracts for the analysis of IAN and IAA were reduced under vacuum at 30°C to a small volume (1 to 4 mL), and then transferred to Sep-Pak C_{18} cartridges pre-conditioned as before. Three 1 mL washes of 0.4% acetic acid were used to load the extracts, and 12-15 mL 80% methanol in 0.4% acetic acid eluted the compounds. This eluate was dried under vacuum, transferred using methanol to a scintillation vial, and subsequently dried under N_2. Methylation was then performed on IAA containing samples as described in 2.2.3. After this, the extract was evaporated to dryness under a N_2 stream in a block heater.

Dry samples were taken up in 1 mL dH_2O and partitioned against diethyl ether: 400 µL of diethyl ether was added, and the sample was mixed briefly and left to stand until the ether and water fractions separated. The ether fraction was transferred to a GC/MS vial, and the procedure repeated twice. After partitioning, the samples were dried in a block heater under a N_2 stream, after which the ether fraction was taken to complete dryness in the sample concentrator (to remove any residual water). Trimethylsilylation was then performed on both IAA and IAN containing samples as described in Chapter 2.2.1. For IAN, the BSTFA (1% TMCS) reacts with the indole nitrogen forming a trimethylsilyl derivative (the derivative is shown in Figure 2.9). For IAA, the diazomethane reacts with the carboxyl group, forming a methyl ester, and the indole nitrogen reacts with the BSTFA (1% TMCS), adding a trimethylsilyl group giving the derivative shown in Figure 2.10.

2.2.7 Indole-3-acetamide

Extracts for the analysis of IAM were reduced under vacuum at 30°C to a small volume (1 to 4 mL), and then purified with Sep-Pak C_{18} cartridges pre-conditioned with 100% methanol followed by dH_2O. Three 1 mL washes of dH_2O was used to load the extract, and 12-15 mL 50% methanol in dH_2O was used elute the hormone. This eluate was dried under vacuum, and transferred using methanol to a scintillation vial, and subsequently dried under N_2. Samples were then acylated using 100 µL of both pentafluoropropionic anhydride and acetonitrile in a 60°C oven for 90 minutes. The indole nitrogen and side-chain amide groups both react with the pentafluoropropionic anhydride to form a bis-pentafluoropropionyl derivative as shown in Figure 2.11.

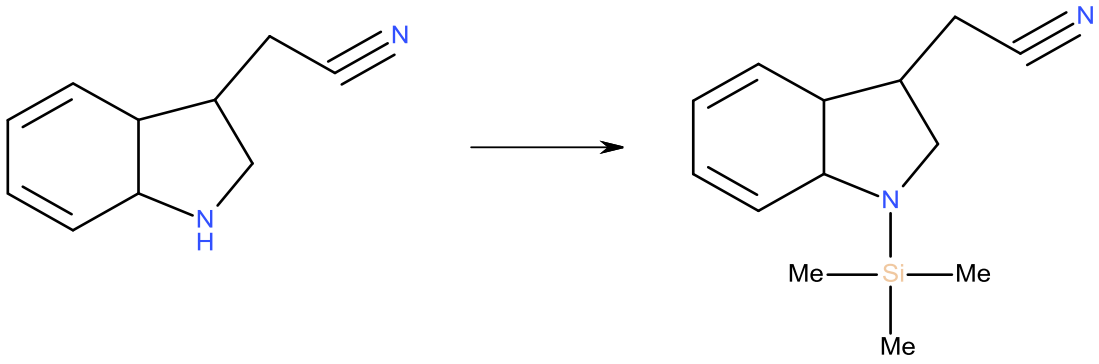

Figure 2.9. The structure of IAN before (left) and after derivatisation with BSTFA (1% TMCS), to form a trimethylsilyl derivative (right).

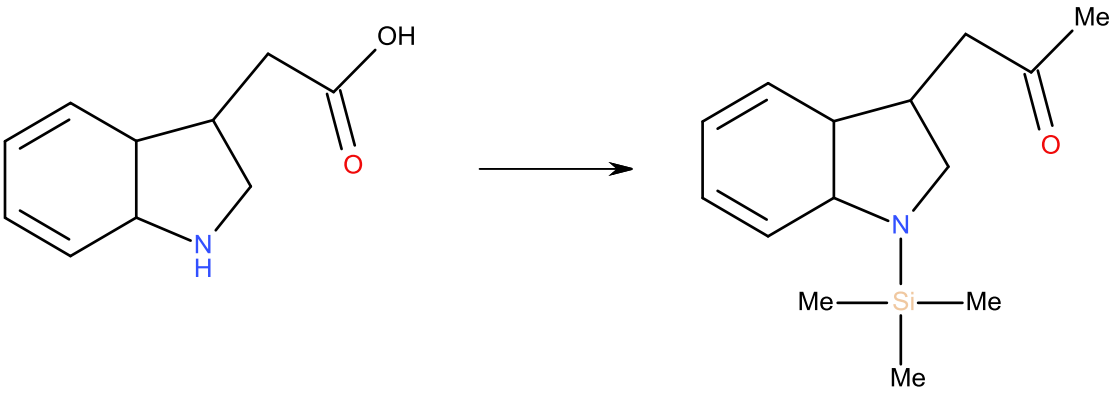

Figure 2.10. The structure of IAA before (left) and after derivatisation with diazomethane and BSTFA (1% TMCS), to form a trimethylsilyl methyl ester derivative (right).

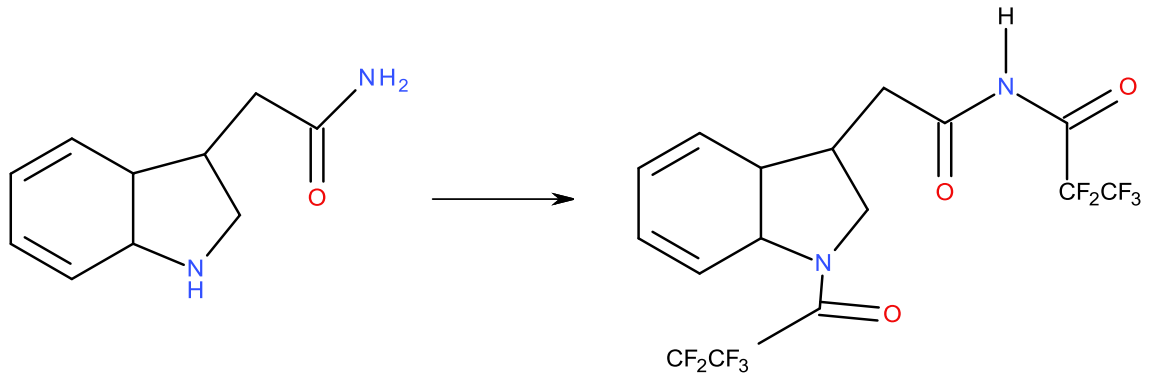

Figure 2.11. The structure of IAM before (left) and after derivatisation with pentafluoropropionic anhydride, to form a bis-pentafluoropropionyl derivative (right).

2.2.8. Final preparation of all samples for GC/MS/MS analysis

All samples were then dried under N_2 and taken up in 50 μL of chloroform to transfer to an autosampler vial for subsequent GC/MS/MS analysis.

2.3. GC/MS/MS analysis

Identification and quantification of the compounds were performed using gas chromatography combined with tandem mass spectrometry. A Varian 3800 GC coupled to Varian 1200 triple quadruple MS was employed, with quantitation via Varian Star software. The column used was a Varian 'Factor Four' VF-5 ms (30 m x 0.25 mm internal diameter and 0.25 micron film), with a Varian 1177 split/splitless injector in splitless mode. The ion source was held at 220°C, the injector 250°C, and the transfer line 290°C, with the typical injection volume being 1 μL. The helium carrier gas was supplied at a constant flow of 1.4 mL per minute. For tryptamine, IEt and IAA the column oven temperature program was 50°C for two minutes, then to 190°C at 30 degrees per minute, then to 220°C at 10 degrees per minute, and finally to 270°C at 30 degrees per minute. For tryptophan the oven temperature program was 50°C for two minutes, then to 270°C at 10 degrees per minute. The GC/MS/MS conditions and specifications for IAA were as described (Jones et al., 2005), and the conditions for all other compounds are shown in Table 2.1.

For unlabelled IAA, the transitions were *m/z* 261 to 202 (-14V collision energy), for [$^{13}C_6$]IAA, *m/z* 267 to 208 (-14V collision energy), and for [$^{2}H_5$]IAA, *m/z* 266 to 207. The parent isolation width used was 2 amu, and the daughter 1 amu, with a cycle time of 0.3 seconds. The retention time was typically 9.85 minutes for IAA.

For tryptamine, IEt, IAOx, IPyA, IAN and tryptophan full scan mass spectra of standards were acquired to determine suitable MS/MS transitions for quantitation. The molecular ion (or in some cases a prominent fragment ion) were selected as the precursor ions. Once relevant product ions were observed, the conditions for quantitation were optimized, including the collision energy for each transition. For unlabelled tryptamine, the transitions were *m/z* 289 to 142 (-22V collision energy), and *m/z* 452 to 289 (-12V collision energy), and for the [$^{2}H_5$]- standard, transitions were *m/z* 294 to 147, (-22V collision energy), and *m/z* 457 to 294 (-12V collision energy). Due to the positions of the deuterium atoms and their partial loss in the MS fragmentations, the [$^{2}H_4$]- standard transitions were *m/z* 292 to 145 (-22V

collision energy) and 456 to 292 (-12V collision energy). The parent isolation width was 2.5 amu, and the product isolation width 2 amu, with a cycle time of 0.3 seconds.

The GC/MS/MS conditions and specifications for IAN were the same as described for IAA, except that the endogenous transition was *m/z* 228 to 73 (-16V collision energy) and 228 to 129 (-10V collision energy), with *m/z* 233 to 73 (-16V collision energy) and 233 to 134 (-10V collision energy) for the [$^2H_5$]IAN. The parent isolation width was 1.2 amu and the product isolation width 1.0 amu, with a cycle time of 0.3 seconds.

For unlabelled IEt (including IAAld converted to IEt using sodium borohydride), the transitions were *m/z* 305 to 202 (-14V collision energy), *m/z* 307 to 202 for the [$^2H_2$]-standard (both deuterium ions were lost in the fragmentation; -14V collision energy), and *m/z* 310 to 207 (-14V collision energy) for the [$^2H_5$]- standard. The parent isolation width was 0.9 amu, and the product isolation width 1.2 amu, with a cycle time of 0.3 seconds.

For unlabelled tryptophan, the transitions were *m/z* 480 to 202 (-10V collision energy), and for the [$^2H_5$]- standard, *m/z* 485 to 207 (-10V collision energy). The parent isolation width was 2 amu and the product isolation width 1.5 amu, with a cycle time of 0.3 seconds.

For unlabelled IPyA, the transitions were *m/z* 484 to 227 (-16V collision energy), and 484 to 287 (-8V collision energy). For [$^2H_5$]IPyA, the transitions were *m/z* 489 to 227 (-16V collision energy), and 489 to 292 (-8V collision energy). The parent isolation width used was 3 amu, and the daughter isolation width 1.5 amu, with a cycle time of 0.3 seconds. The column oven temperature program was 50°C for 2 minutes, then to 240°C at 30 degrees per minute, then to 280°C at 10 degrees per minute. Retention times of the two isomers were 0.18 minutes apart for IPyA.

For unlabelled IAOx, the transitions were *m/z* 228 to 73 (-16V collision energy), and 318 to 228 (-10V collision energy). For [$^2H_5$]IAOx, the transitions were *m/z* 233 to 73 (-16V collision energy), and 318 to 228 (-10V collision energy). The parent isolation width used was 2 amu, and the daughter 2 amu, with a cycle time of 0.3 seconds.

Table 2.1. GC/MS/MS specifications for unlabelled and labelled versions of tryptophan, tryptamine, IAN, IEt, IAOx, IAM and IAA. The derivative used for analysis is shown for each compound, as well as the ion transitions, and the parent and daughter isolation widths used for analysis.

Compound	Abbreviation	Derivative[A]	Parent and daughter ions (*m/z*)	Parent isolation width (amu)	Daughter isolation width (amu)
Tryptophan	tryptophan	EC, TMS, PFPropyl	480 to 202 (-10V)	2	1.5
[$^2H_5$]tryptophan	[$^2H_5$]tryptophan	EC, TMS, PFPropyl	485 to 207 (-10V)	2	1.5
[^{14}C]tryptophan	[^{14}C]tryptophan	EC, TMS, PFPropyl	482 to 204 (-10V)	2	1.5
Tryptamine	Tryptamine	Bis-PFP	289 to 142 (-22V) 452 to 289 (-12V)	2.5	2
[$^2H_5$]tryptamine	[$^2H_5$]tryptamine	Bis-PFP	294 to 147 (-22V) 457 to 294 (-12V)	2.5	2
[$^2H_4$]tryptamine[B]	[$^2H_4$]tryptamine	Bis-PFP	292 to 145 (-22V) 456 to 292 (-12V)	2.5	2
[^{14}C]tryptamine	[^{14}C]tryptamine	Bis-PFP	291 to 144 (-22V) 454 to 291 (-12V)	2.5	2
Indole-3-acetonitrile	IAN	TMS	228 to 73 (-16V) 228 to 129 (-10V)	1.2	1
[$^2H_5$]Indole-3-acetonitrile	[$^2H_5$]IAN	TMS	233 to 73 (-10V) 133 to 134 (-10V)	1.2	1
Indole-3-ethanol[C]	IEt	Bis-TMS	305 to 202 (-14V)	0.9	1.2
[$^2H_2$]indole-3-ethanol	[$^2H_2$]IEt	Bis-TMS	307 to 202 (-14V)	0.9	1.2
[$^2H_5$]indole-3-ethanol	[$^2H_5$]IEt	Bis-TMS	310 to 207 (-14V)	0.9	1.2
Indole-3-acetaldoxime	IAOx	Bis-TMS	228 to 73 (-16V) 318 to 228 (-10V)	2	2
[$^2H_5$]indole-3-acetaldoxime	[$^2H_5$]IAOx	Bis-TMS	233 to 73 (-16V) 323 to 233 (-10V)	2	2
Indole-3-acetic acid	IAA	ME, TMS	261 to 202 (-16V)	2	2
[$^2H_5$]indole-3-acetic acid	[$^2H_5$]IAA	ME, TMS	266 to 207 (-16V)	2	2
Indole-3-acetamide	IAM	Bis-PFP	276 (GC/SIM/MS)	1.5	1.5
[$^2H_6$]indole-3-acetamide	[$^2H_6$]IAM	Bis-PFP	282 (GC/SIM/MS)	1.5	1.5
Indole-3-pyruvic acid	IPyA	PFB, ME, TMS	484 to 227 (-16V) 484 to 287 (-8V)	3	1.5
[$^2H_5$]indole-3-pyruvic acid	[$^2H_5$]IPyA	PFB, ME, TMS	489 to 227 (-16V) 489 to 292 (-8V)	3	1.5

[A]Abbreviations for derivatives are as follows: EC – N-ethoxy carbonyl; TMS – N-trimethylsilyl; PFPropyl – pentafluoropropyl ester; Bis-PFP – N,N-Bis-pentafluoropropionyl; ME – methyl ester; PFB - pentafluorobenzylhydroxylamine. [B]Standard transitions were altered due to the positions of the deuterium atoms and their partial loss in the MS fragmentations. [C]Including IAAld converted to IEt using sodium borohydride.

For both [$^2H_6$]IAM and unlabelled IAM, GC/selected ion monitoring/MS (GC/SIM/MS) was used for analysis. The GC conditions were the same as for IAA. The ions monitored were *m/z* 276 for unlabelled IAM, *m/z* 281 for [$^2H_5$]IAM, and *m/z* 282 for [$^2H_6$]IAM. The peak width was set to 1.5 amu, with a cycle time of 0.3 seconds.

Compounds were all separable by GC/MS/MS, and eluted in the order IAM, followed 1.09 minutes later by tryptamine, then followed 0.6 minutes later by IAN, then (three seconds) IEt, (eight seconds) IAA, (0.88 minutes) IAOx, then followed by the two IPyA isomers, the first after 1.06 minutes, and the next 0.17 minutes later, and then finally followed 0.38 minutes later by tryptophan.

Compounds were identified on the basis of retention times and by monitoring a selected transition (or two transitions when possible) characteristic for the compound in question (as shown for each compound in Table 2.1). Calculations of endogenous levels were performed by comparing peak areas of a transition derived from the endogenous hormone and the corresponding stable-isotope-labelled internal standard (Figure 2.12). To calculate the endogenous hormone level, the corrected endogenous product ion intensity (peak area) was divided by the internal standard product ion intensity (peak area), and this was then multiplied by the amount of internal standard added, divided by the fresh weight of the tissue, used to give results in ng $g(FW)^{-1}$. Corrections were made for the small amount of unlabelled material in the deuterated standards, and for contributions from natural isotopes (of [^2H]- and [^{13}C]-) in unlabelled material to peaks corresponding to internal standards (as shown for each compound in Table 2.2). For measurement of endogenous IAAld after its conversion to IEt, the endogenous IEt was subtracted from the total IEt, after the conversion was complete.

2.4. Statistical Analysis

Statistical analysis was performed on most data sets – used was a student's t-test, where the mean value of one treatment was subtracted from the mean value of the second treatment. This figure was then divided by the square root of the sum of the standard errors for the two treatments, squared. The degrees of freedom were calculated as $(n_1 + n_2) - 2$. A probability of less than 0.05 was deemed significant.

$$\text{Endogenous level ng g(FW)}^{-1} = \left(\frac{\text{Corrected endogenous peak area}}{\text{Corrected internal standard peak area}}\right) \times \left(\frac{\text{ng internal standard added}}{\text{fresh weight (FW)}}\right)$$

Figure 2.12. Calculation for the quantification of endogenous hormone or precursor level in ng g $(FW)^{-1}$. Corrections were made for the contribution of deuterium to the endogenous channel, as well as heavy isotopes of endogenous compounds.

Table 2.2. The composition of synthesised IAA precursors. The percentage and form of label in synthesised deuterated IAA precursors tryptophan, tryptamine, IAN, IEt, IAOx and IPyA, as determined by GC/MS/MS (or GC/SIM/MS for IAM). Data are based on uncorrected peak areas. The shaded areas are not relevant to the study. Note: the [$^2H_6$]IAM was used subsequently in quantitation and metabolism studies analysed by LC/MS, as described in Chapter 6.

	tryptophan	**tryptamine**	**IAN**	**IEt**	**IAOx**	**IAM**	**IPyA**
[$^2H_8$]						23.84%	
[$^2H_7$]						37.04%	
[$^2H_6$]						38.25%	
[$^2H_5$]	71.00%	59.35%	85.00%		93.9%	0.86%	56.65%
[$^2H_4$]	18.37%	33.94%	7.80%		6.14%		27.44%
[$^2H_3$]	4.76%	5.38%	0.33%		0%		8.41%
[$^2H_2$]	3.36%	0.82%	0.14%	98.96%	0%		3.66%
[$^2H_1$]	1.99%	0.38%	0.66%	0.95%	0%		2.47%
unlabelled	0.27%	0.13%	6.00%	0.09%	0%	0%	1.37%

CHAPTER 3

Development of Methodologies

3.1. Introduction

In order to correctly identify and quantify the intermediates outlined in Chapter 2, it was necessary to develop and optimise the methods by which the IAA precursors discussed were prepared for analysis. This chapter outlines the development or optimisation of methodologies for the isolation of tryptophan, tryptamine, IEt, IAOx and IAN, and the quantification of IAAld. This chapter also describes the development of a technique for aseptically growing pea seedlings, and conducting metabolism studies in a sterile environment.

3.2. Method development and results

3.2.1. Tryptophan

Perrine et al. (2004) have shown that derivatisation of tryptophan by pentafluoropropanol for GC/MS analysis was successful in *Rhizobium* cultures. This was confirmed using standard unlabelled DL-tryptophan (Sigma-Aldrich, St. Louis, MO, USA). In order to translate this to pea plant extract analysis, plants were harvested in wholly aqueous conditions, and the described derivatisation techniques were performed. After this, extracts were partitioned against chloroform, and the organic phase dried under nitrogen (as described in Chapter 2.2.1). After GC/MS/MS analysis of this extract, it was found that a further derivatisation step, trimethylsilylation, allowed sharper peaks, and clearer mass spectrometry (Figure 3.1).

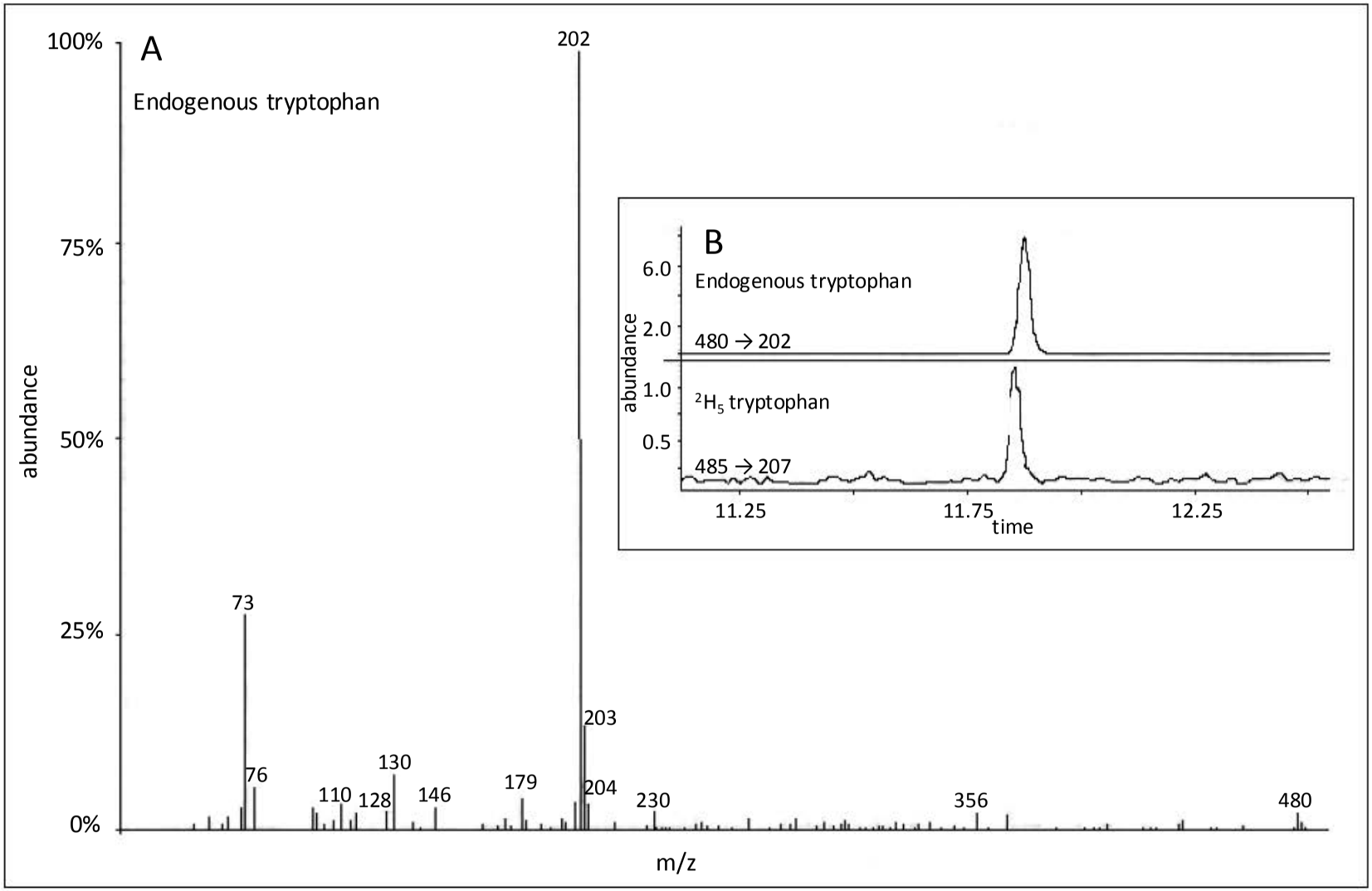

Figure 3.1. Tryptophan isolation in pea. **A**: Mass spectrum of genuine endogenous tryptophan. **B**: Endogenous tryptophan and pea tissue (top), and [$^2H_5$]tryptophan internal standard (bottom).

3.2.2. Tryptamine

Quittenden (2005) subjected plant extracts to Sep-Pak C_{18} cartridge purification and HP/LC analysis before using pentafluoropropionic anhydride to derivatise before GC/MS/MS analysis. This method is expensive and time consuming. It was found that after drying, extracts could be resuspended in 2% acetic acid in dH_2O, and partitioned against chloroform for purification. Subsequently, the inorganic phase could be taken to pH 11 using 1N NaOH, and partitioned against chloroform again. Tryptamine passed into the chloroform on this partition step, which was dried and pentafluoropropionic anhydride used to derivatise the compound, with the length of time heated at 60˚C raised from 60 to 90 minutes (as described in Chapter 2.2.2). This was confirmed using standard unlabelled tryptamine (Sigma-Aldrich, St. Louis, MO, USA). As described in Quittenden et al. (2009), this is the first identification and quantification of tryptamine in pea tissue (Figure 3.2).

3.2.3. IAN

Again, although Barlier et al. (2000) had developed techniques for the purification, derivatisation and analysis of IAN in *Arabidopsis thaliana* tissue by GC/MS/MS, the purification techniques needed to be adapted to suit pea tissue. Because of the similarity in structure of IAN to IAA, synthetic standards of the compound could be purified using standard IAA techniques: Sep-Pak C_{18} cartridge purification, followed by diethyl ether partitioning, and subsequent trimethylsilylation (as described in Chapter 2.2.6). However, some plant extracts required further purification by HP/LC. In order to separate IAA and IAN on this system, a gradient of 20 to 60% methanol in 0.4% acetic acid was used over 40 minutes, with a five minute wait period, collecting 2 mL per minute. Unlabelled synthetic IAN eluted after 20-22 minutes. Although these techniques recovered both deuterium labelled and unlabelled IAN standards (unlabelled obtained from Merck, Hohenbrunn, Germany; [$^2H_5$]IAN from Jason Smith, School of Chemistry, UTAS), when used for internal standard purposes, no dilution of [$^2H_5$]-labelled IAN was seen in pea plant samples: that is, endogenous IAN was not found to be present in pea tissue. However, the compound was found in *Arabidopsis thaliana* shoot tissue (Figure 3.3).

3.2.4. IAOx

Repeated trials using plant extracts spiked with synthetic IAOx standards (obtained from Jason Smith, School of Chemistry, University of Tasmania, Australia) found that purification by Sep-Pak C_{18} cartridges and HP/LC analysis were required before derivatisation by

trimethylsilylation for the recovery of synthetic IAOx (as described in Chapter 2.2.4). Again, these techniques repeatedly recovered synthetic internal standards added to plant extracts, but no dilution of [$^2H_5$]-labelled IAOx was shown in any plant extract: no endogenous IAOx was found in pea tissue.

3.2.5. IEt and IAAld

It has been previously shown that the levels of IAAld can be measured in pea tissue by converting the compound to IEt using sodium borohydride (Moore & Shaner, 1968; Quittenden, 2005; as confirmed using unlabelled synthetic IEt (obtained from Jason Smith, School of Chemistry, University of Tasmania, Australia)). However, this crude method had not been optimised, and needed further investigation. Quittenden (2005) used 200 mg sodium borohydride to convert IAAld to IEt in plant extracts magnetically stirred for one hour. The extracts were then purified using Sep-Pak C_{18} cartridges, and HP/LC analysis. This technique was lengthy and expensive to complete.

In order to further develop this technique, pea plant extracts were harvested as described (Chapter 2.1.1), and used for a number of trials. Firstly, adding only 200 mg of sodium borohydride, and stirring the extract over 1-5 hours resulted in very large standard errors between the replicates of the level of IAAld (converted to IEt) from the same extract (Figure 3.4). It appeared as though more IAAld (converted to IEt) was recovered after 5 hours than 1-4 hours. Next, aliquots of the extract spiked with 100 ng IAAld (sodium bisulfite addition compound, Sigma-Aldrich, St. Louis, MO, USA) were magnetically stirred or shaken overnight (18 hours) with 200 mg sodium borohydride. The difference between methods for mixing the sodium borohydride by stirring and shaking were very large – with the resulting IAAld (IEt) at ~11 ng g $[FW]^{-1}$ for the shaken, and ~110 ng g $[FW]^{-1}$ for the stirred (Figure 3.5). Therefore, stirring the solutions with a magnetic stirrer was more efficient.

Subsequently, one gram of sodium borohydride was added to aliquots of the same pea extract, and stirred over a period of ten hours. For every hour after five hours, one sample was taken to check for the level of IAAld converted to IEt (Figure 3.6). It was found that there was a noticeable difference between the level of IEt in the resulting solution after 9 hours in comparison to 8 hours stirring, but not between 9 and 10 hours. It was deemed that 9 hours would be an appropriate amount of time to stir the extracts for analysis of IAAld (converted to IEt).

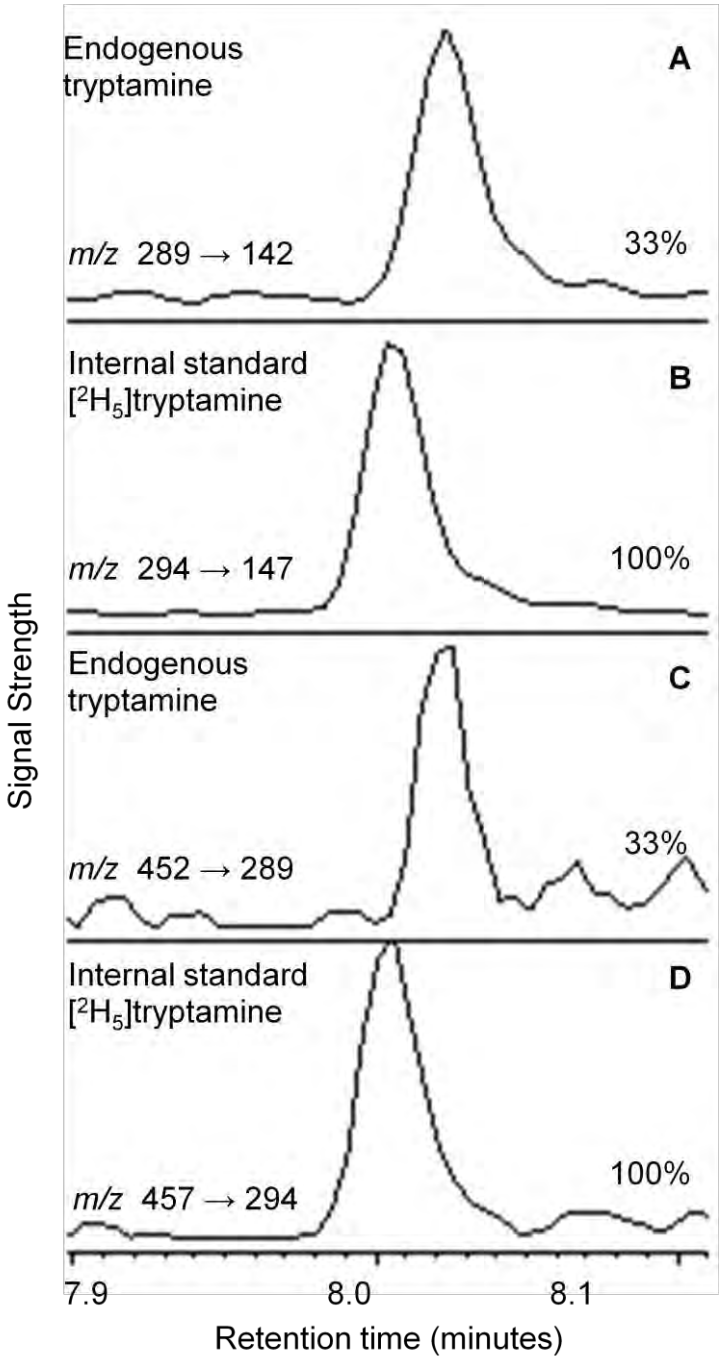

Figure 3.2. The identification and quantification of tryptamine. GC/MS/MS chromatograms showing (derivatised) endogenous tryptamine (A, C) and internal standard [$^2H_5$]tryptamine (B, D) peaks in an extract from pea roots. Due to the very low levels of tryptamine endogenously, no mass spectral identification was possible. As for other compounds, the presence of deuterium reduces the retention time of tryptamine.

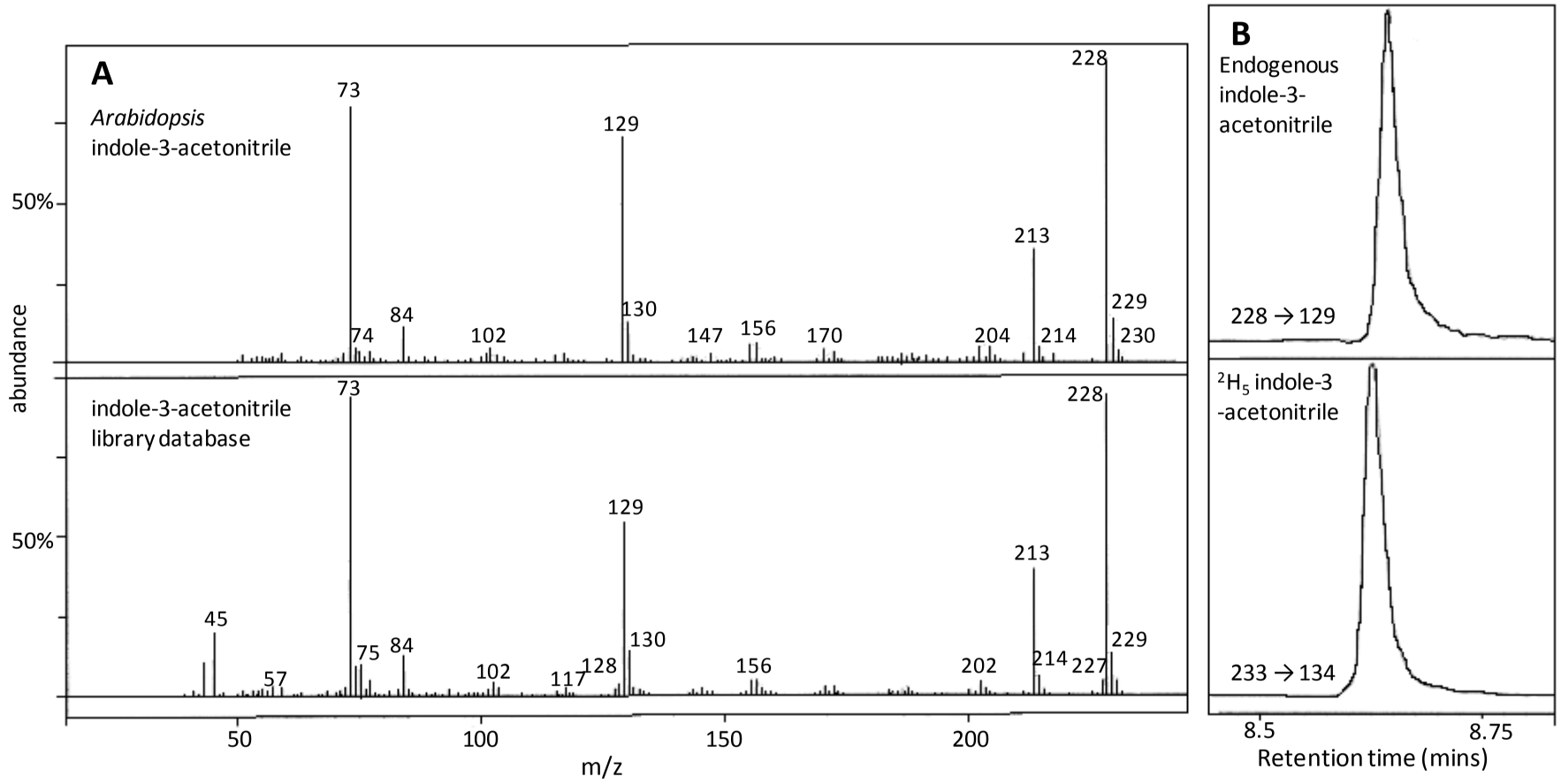

Figure 3.3. IAN in *Arabidopsis thaliana*: **A**: Mass spectrum of *Arabidopsis* IAN (top) and genuine IAN (bottom) from MS library database**.** **B**: GC/MS-MS chromatogram of internal standard [$^{2}H_5$]IAN (bottom) and endogenous IAN (top) in *Arabidopsis thaliana* tissue. This could not be found in pea tissue.

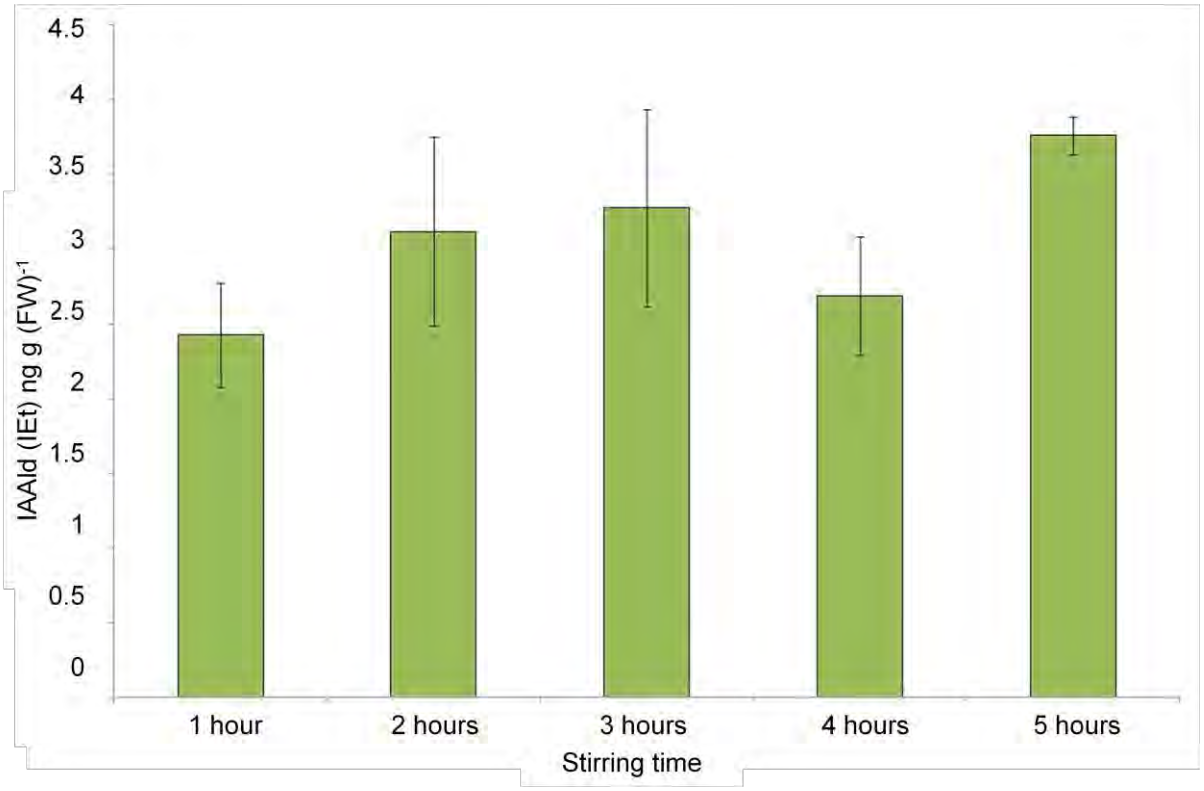

Figure 3.4. The level of IAAld converted to IEt over one to five hours after a pea extract was magnetically stirred with 200 mg sodium borohydride. Shown are the means ± standard errors (n=2).

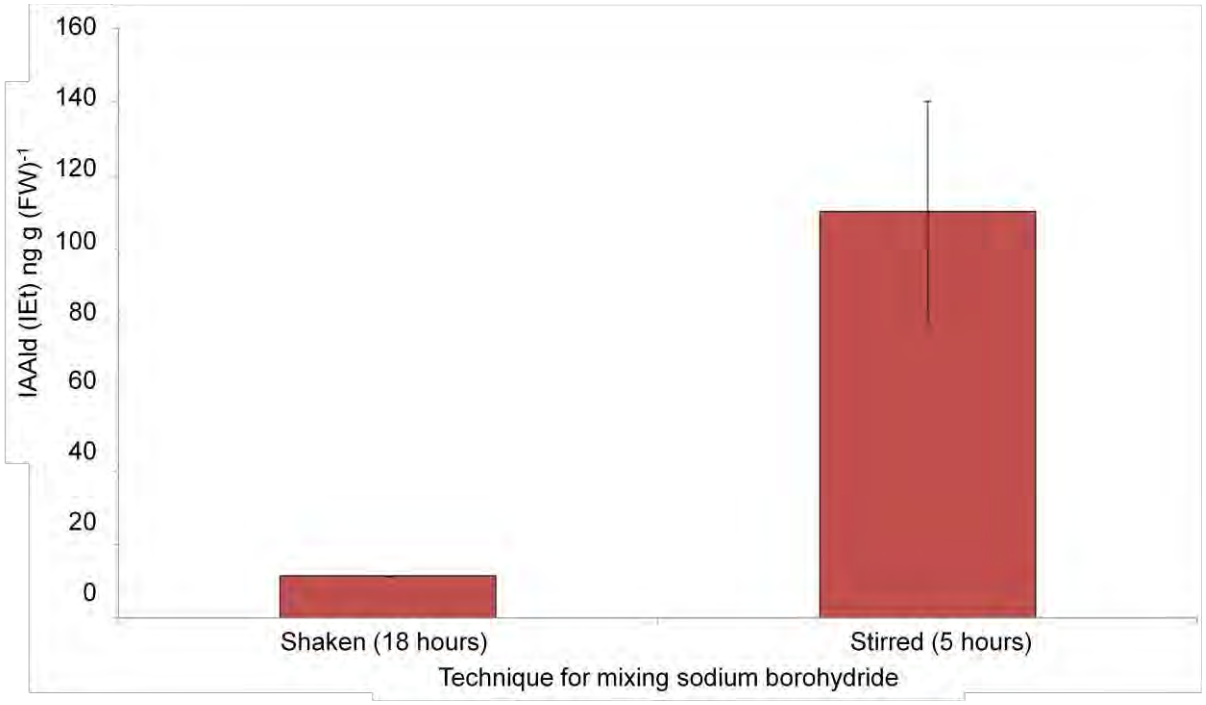

Figure 3.5. The level of IAAld converted to IEt after aliquots of a plant extract spiked with 100 ng of synthetic IAAld were left on a shaker for 18 hours, or stirred magnetically for five hours, with 200 mg sodium borohydride. Shown are the means ± standard errors (n=2).

After this, the amount of sodium borohydride needed for full conversion of IAAld to IEt was tested. Aliquots of the same pea extracts were stirred for five hours with either 600 mg of sodium borohydride added once, or 600 mg added three times, or for 9 hours with either 600 mg added twice, or one gram of the reagent added twice. It was found that even though more sodium borohydride was added in the five hour treatment with three additions of 600 mg, significantly more IAAld was converted to IEt in the nine hour treatment with two additions of one gram sodium borohydride (Figure 3.7). This time frame and level of sodium borohydride was thus used for IAAld analysis forthwith (as described in Chapter 2.2.5).

The resulting extracts were difficult to purify due to the excess of reagent present. Therefore, rather than using Sep-Pak and HP/LC purification, it was found that the excess reagent in vacuum-dried plant samples could be reacted with $KHSO_4$ (0.3 N), and IEt extracted into chloroform on partitioning against this solvent. The chloroform could then be dried by vacuum, and trimethylsilylation preformed as before in order to analyse the compound by GC/MS/MS. Samples for the analysis of IEt and IAAld (converted to IEt) were treated to the same purification and derivatisation, over the same time period to standardise results.

3.2.6. Metabolism studies and aseptic growth

The technique for placing roots in aerated water for metabolism studies was adapted from Weston et al. (2009), however, the system needed to be kept aseptic to allow uptake of labelled compounds into the roots not being converted by bacteria (Weston et al. grew plants in soil prior to placing in 1.3% agar [containing MS] with the compound of interest). Therefore, plants were grown aseptically (in MS media as described in Chapter 2.1.2), and after one week, were removed from growth tubes, and the roots placed in autoclaved aerated water with the compound of interest. This was all conducted in a lamina flow cabinet. The tubes were left overnight in a lamina flow cabinet for sterility, with a hood to protect from desiccation (Figure 3.8) as described in Chapter 2.1.2.

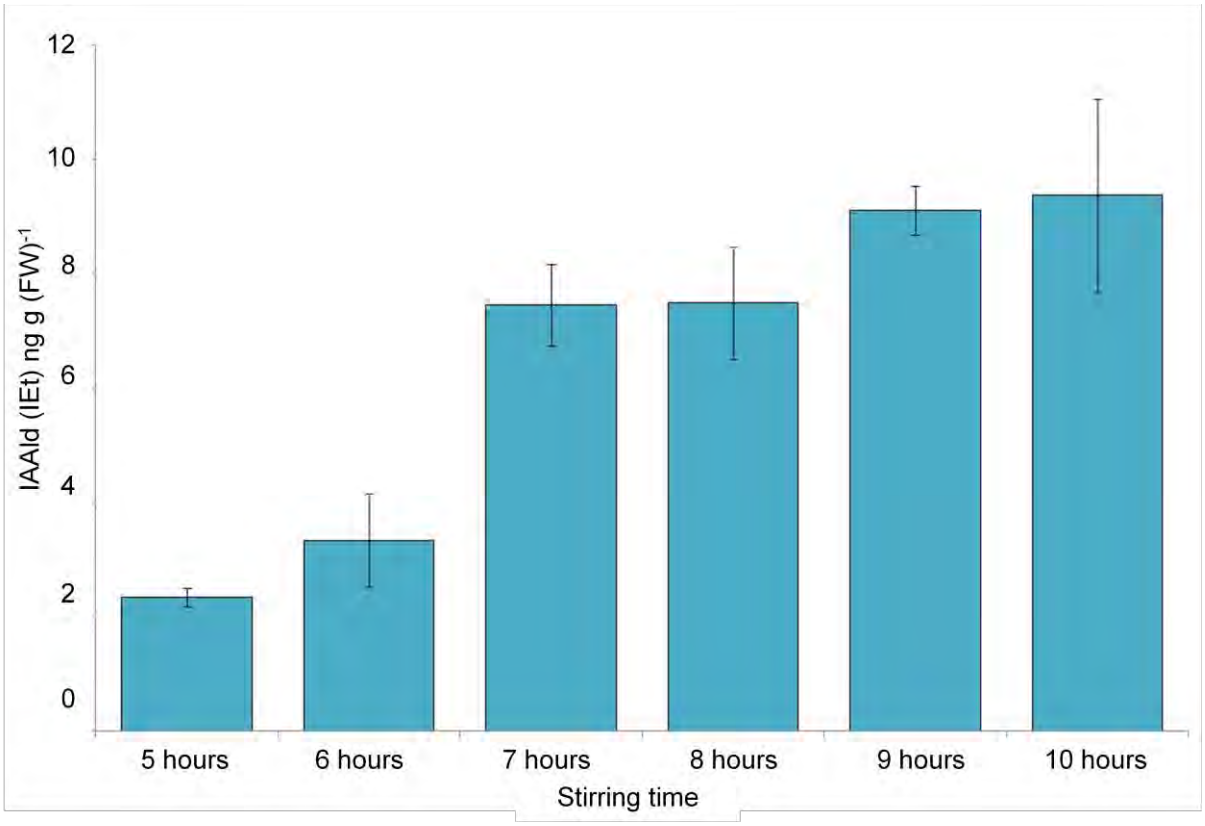

Figure 3.6. The level of IAAld converted to IEt over five to ten hours after a pea extract was stirred with 1 g sodium borohydride. Shown are the means ± standard errors (n=2).

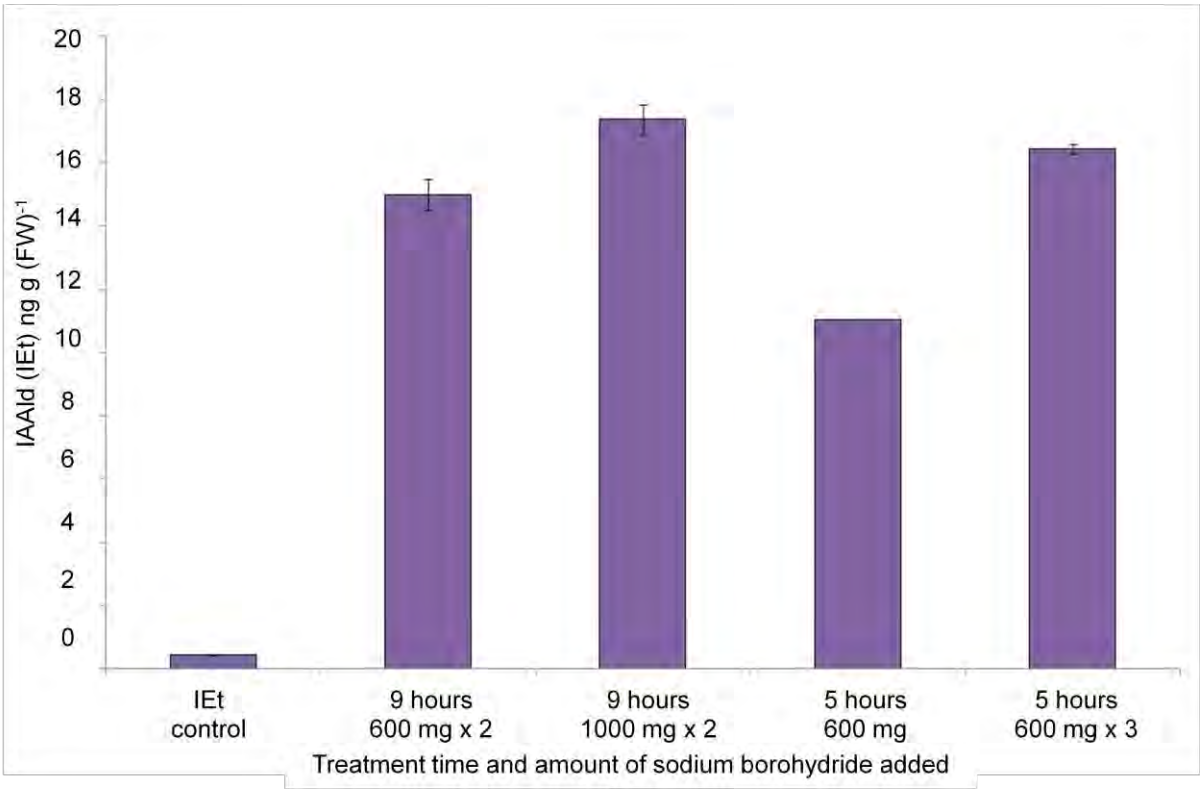

Figure 3.7. The amount of IAAld converted to IEt from left: base level of IEt in plant extract; extract stirred for nine hours, with 600 mg sodium borohydride added twice; extract stirred for nine hours, with 1 g sodium borohydride added twice; extract stirred for five hours with 600 mg sodium borohydride added once, and; extract stirred for five hours with 600 mg sodium borohydride added three times. Shown are the means ± standard errors (n=2).

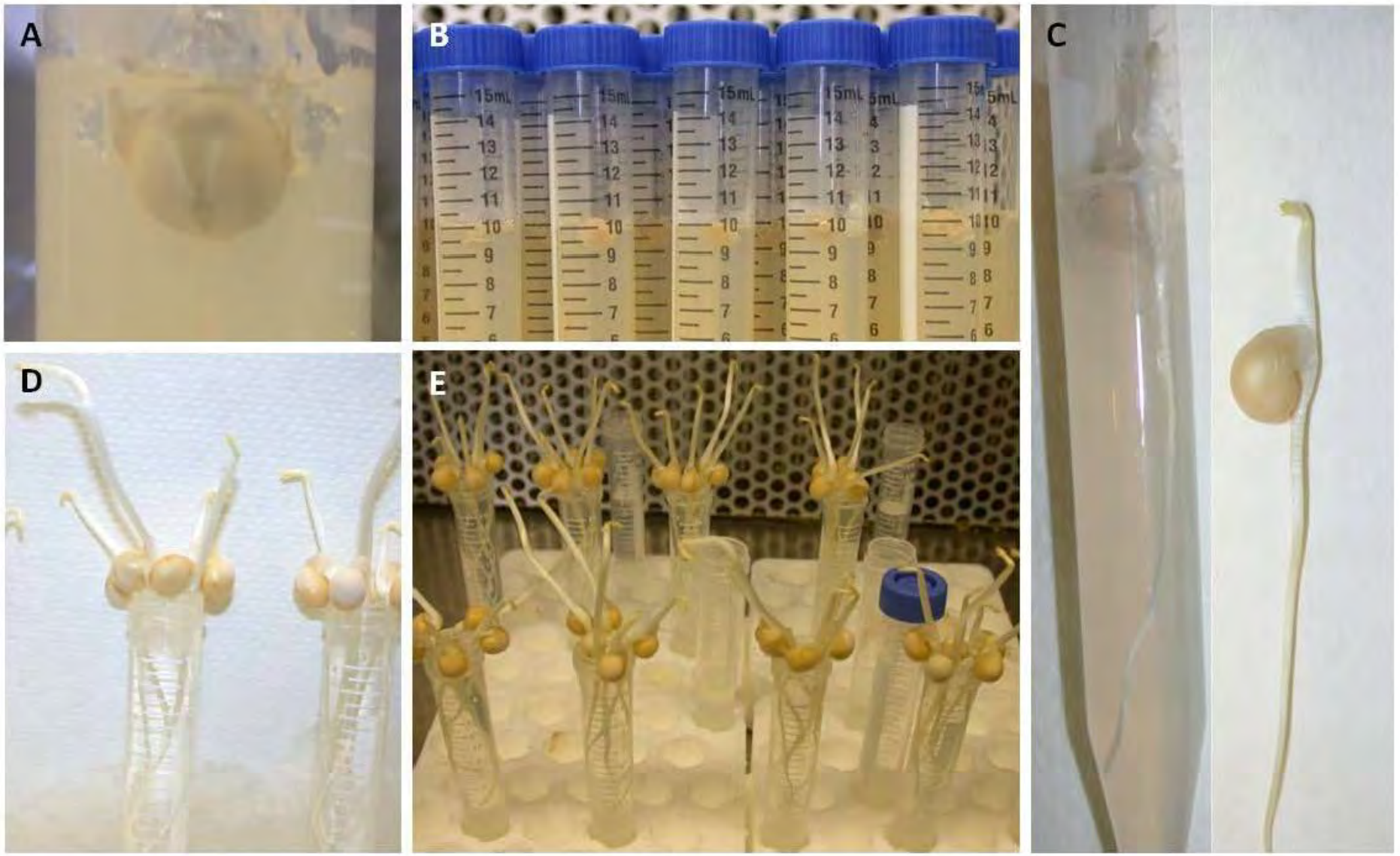

Figure 3.8. The process of growing plants aseptically in order to conduct metabolism studies. **A** and **B**: sowing of pea seeds in agar with MS medium – with the radicle facing down to promote straight root growth. **C**: After one week, seedlings are removed from agar, and any residue washed off. **D** and **E**: washed seedlings are placed in tubes containing aerated sterile water, along with the labelled compound of interest – generally 4 seedlings to each tube, and left over night in a lamina flow cabinet (with a hood to protect the plants from dessication) to insure aseptic conditions.

3.3. Discussion

The development of techniques for the isolation and quantification of IAA precursors is vital to the investigation of IAA biosynthesis from a physico-chemical perspective. Herein, GC/MS/MS techniques for the successful analysis of the IAA precursors tryptophan, tryptamine, IAN, IAOx, IAAld and IEt have been outlined. The development of techniques for the isolation of IPyA and IAM are outlined in Chapters 5 and 6 consecutively. Furthermore, a technique of germinating and growing pea seedlings in agar with MS medium has been developed, along with a successful technique for monitoring uptake of deuterium labelled precursors into roots from whole seedlings. These techniques may be of great importance to any further experimentation involving the metabolism of known biosynthesis-related compounds with a requirement of a sterile *in vivo* system.

CHAPTER 5

The Indole-3-Pyruvic Acid Pathway to IAA

5.1. Introduction

IPyA has long been implicated as an intermediate in IAA biosynthesis (Nonhebel et al., 1993). However, evidence to support the compound's involvement has recently been focussed on enzymes required for its production, and relatively few studies have involved the isolation or quantification of the actual compound. It is a presumption that IPyA is widely abundant in plants, with its occurrence having been noted only in tomato (Cooney and Nonhebel, 1989; Cooney and Nonhebel, 1991), and *Arabidopsis* (3-13 ng g $(FW)^{-1}$; Tam and Normanly, 1998) using modern techniques (such as GC/MS), and also in barley using out-dated techniques (Gibson et al., 1972b). The compound has also been found in legume-root nodulating bacteria such as *Bradyrhizobium elkanii* (Minamisawa et al., 1996) as well as in *Rhizobium* nodules of pea (Badenoch-Jones et al., 1984; Carreño-Lopez et al., 2000).

The IPyA pathway to IAA involves the conversion of tryptophan to IPyA by an aminotransferase (Zazimalova and Napier, 2003), and the subsequent conversion of IPyA to IAAld by a decarboxylase (Nonhebel et al., 1993; Figure 5.1). Enzymes specific for the transamination of tryptophan to IPyA have been found in species of 16 different families (Truelsen, 1973), ranging from mung bean (Wightman and Cohen, 1968), maize (Kutáček, 1985), pea (Moore and Shaner, 1968), white lupin (Wilson et al., 1954) and tomato and barley segments (Gibson et al., 1972b). Furthermore, tomato and *Avena* plants incubated with radiolabelled tryptophan showed an accumulation of radioactivity co-eluting with IPyA by thin layer chromatography (Schneider et al., 1970; Gibson et al., 1972b). However, again some of the techniques used to gather this evidence are outdated, and not specific enough to be unequivocal.

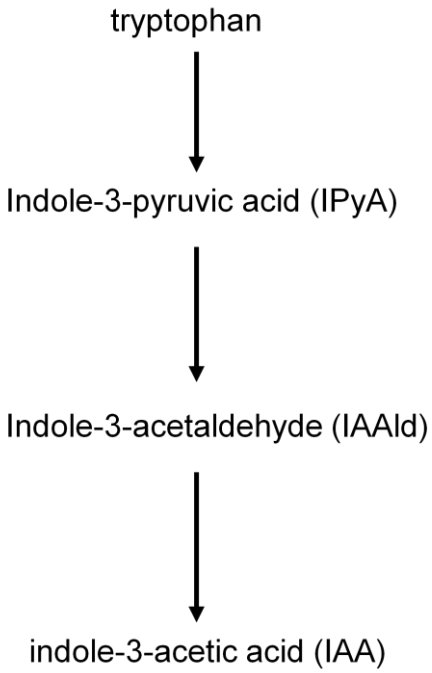

Figure 5.1. The IPyA pathway to IAA proceeds from tryptophan to IPyA by an aminotransferase, and then to IAAld by decarboxylase, which is then converted to IAA by aldehyde oxidase. Adapted from Woodward and Bartel (2005).

Recently, Tao et al. (2008) and Stepanova et al. (2008) discovered allelic genes that encode an *Arabidopsis* tryptophan aminotransferase (*TAA1*). The genes were originally implicated in shade avoidance responses, and ethylene sensitivity. However, it was soon uncovered that the ethylene response was a product of IAA biosynthesis in specific tissues and cell types. When *TAA1* was expressed in *E. coli*, the induced protein converted tryptophan to IPyA (confirmed by LC/MS/MS analysis; Stepanova et al., 2008; Tao et al., 2008). These recent findings brought IPyA back into the IAA biosynthesis spotlight.

The enzyme IPyA decarboxylase, which converts IPyA to IAAld, has been isolated in the bacteria *Bradyrhizobium elkanii* (Minamisawa et al., 1996; Patten and Glick, 1996), and the genes encoding the enzyme were isolated in the nitrogen fixing bacteria *Enterobacter cloacae* (Koga et al., 1991) and *Azospirillum brasilense* (Costacurta et al., 1994; Carreño-Lopez et al., 2000). However, the involvement of this enzyme in higher plants is not known, despite the finding that the application of IPyA to decapitated pea plants resulted in an auxin-like inhibition of lateral bud outgrowth (Nakajima et al., 2002). There is a gap in the research relating to IPyA in that few studies have examined the presence of the compound, or the metabolism of labelled versions of IPyA using modern techniques.

Herein, the IPyA pathway is investigated in pea. The following experiments use GC/MS/MS in order to analyse synthetic IPyA, and test the stability of the compound in both derivatised and non derivatised forms. Incubation studies are also conducted using [$^2H_5$]tryptophan and [$^2H_5$]IPyA in order to analyse their conversion to [$^2H_5$]IPyA and [$^2H_5$]IAA, respectively.

5.2. Materials and Methods

5.2.1. The production of [$^2H_5$]IPyA from synthetic [$^2H_5$]tryptophan

Approximately 200 µg of [$^2H_5$]tryptophan was dissolved in 0.2 mM potassium phosphate buffer (pH 7.6) containing 500 units of catalase (900 units/mg; Sigma-Aldrich, St. Louis, MO, USA). Two units of L-amino acid oxidase (from *Crotalus atrox* venom; Sigma-Aldrich, St. Louis, MO, USA; 1-3 units/mg) were added and the mixture was incubated at 37°C for 1 hour (as described by Lam et al., 1990). The mixture was acidified to pH 3 and extracted three times against 10 mL ethyl acetate. The ethyl acetate phase was dried under N_2, and then stored at -20°C until use. The converted sample was prepared for analysis for [$^2H_5$]tryptophan and [$^2H_5$]IPyA (as confirmed with unlabelled IPyA obtained from -Aldrich, St. Louis, MO,

USA) as described in Chapters 2.2.1 and 2.2.3, and subsequently tested by GC/MS/MS (Chapter 2.3).

5.2.2. The analysis of synthetic, and endogenous pea IPyA and internal standard [$^2H_5$]IPyA using GC/MS/MS

Both synthetic unlabelled and [$^2H_5$]IPyA, as well as plant samples (harvested as described in Chapters 2.1 and 2.1.1) with [$^2H_5$]IPyA as an internal standard were purified and derivatised as described in Chapter 2.2.3. The extracts were then subjected to GC/MS/MS analysis for IPyA as described in Chapter 2.3.

5.2.3. An investigation into the stability of IPyA

Because of the loss of the internal standard within plant samples, the stability of IPyA was tested. [$^2H_5$]IPyA was analysed for the presence of [$^2H_5$]IAA by GC/MS/MS conditions described (using [$^{13}C_6$]IAA as an internal standard; Chapter 2.3). Furthermore, two extracts of 200 ng of IPyA, with 20 ng [$^2H_2$]IEt as an internal standard were derivatised as described (2.2.3). One extract (A) was analysed by GC/MS/MS initially (as described in Chapter 2.2), and then after 72 hours and 96 hours (leaving the sample at room temperature to evaporate by one third and then one half, respectively). The other extract (B) was left at room temperature for 96 hours, analysed, and then left for 10 days, and analysed again.

5.2.4. The metabolism of [$^2H_5$]IPyA to[$^2H_5$]IAA in pea roots

A [$^2H_5$]IPyA metabolism study using 10 μM [$^2H_5$]IPyA (as produced in Chapter 5.2.1) was conducted as described in Chapter 2.2.1, and pea root samples were extracted, filtered, purified and derivatised for unlabelled and [$^2H_5$]IAA, as described in Chapter 2.2.6.

5.2.5. The metabolism of [$^2H_5$]tryptophan to [$^2H_5$]IPyA in pea roots

A [$^2H_5$]tryptophan metabolism study was conducted as described in Chapter 2.2.1, and pea root samples were extracted, filtered, purified and derivatised for unlabelled and [$^2H_5$]IPyA, as described in Chapter 2.2.3.

5.3. Results

5.3.1. Analysis of IPyA synthetic standards, and an investigation into [$^2H_5$]IPyA stability

A synthetic standard of 500 ng IPyA was successfully analysed by GC/MS/MS. However, the IPyA signal appeared to be approximately 1000 times less than expected. The production of

[$^2H_5$]IPyA from [$^2H_5$]tryptophan was also successful, with only 0.266% [$^2H_5$]tryptophan found in the putative [$^2H_5$]IPyA. However, the levels of the [$^2H_5$]IPyA observed in the subsequent solution were low, and appeared to become lower over the period of one week (as shown for 500 ng IPyA and 50 ng [$^2H_5$]IPyA in Figures 5.2 and 5.3).

No evidence for IPyA was found within any plant sample; however, this analysis did show that the 50 ng [$^2H_5$]IPyA internal standard (produced one week previously) added before purification and derivatisation of plant extracts was lost prior to GC/MS/MS analysis being conducted (Figure 5.4). On further investigation, when 500 ng of synthetic IPyA and 50 ng [$^2H_5$]IPyA was left non derivatised for one week at -20°C, there was no evidence for [$^2H_5$]IPyA remaining. However, the solution did contained a large amount of both unlabelled and [$^2H_5$]IAA (Figure 5.5).

Consequently, the standards were tested for stability after derivatisation. Using GC/MS/MS analysis, and 20 ng [$^2H_2$]IEt as an internal standard, no noticeable loss of the 200 ng IPyA was seen between the initial analysis (extract A), and after 10 days (extract B; Table 5.1). However, it was noted that [$^2H_2$]IEt itself was being lost, based on a weakening of signal in the correct ion channel. It appears as though IPyA is, in fact, reasonably stable after derivatisation.

5.3.2. The metabolism of [$^2H_5$]IPyA to [$^2H_5$]IAA in pea roots

A metabolism study was conducted to study the metabolic fates of [$^2H_5$]IPyA *in vivo*. However, analysis of root extracts indicated that [$^2H_5$]IPyA was not successfully taken up into the plant roots, or if it was, it was degraded to IAA prior to the uptake, and the result was therefore deemed equivocal (data not shown).

5.3.3. The metabolism of [$^2H_5$]tryptophan to [$^2H_5$]IPyA in pea roots

A metabolism study was also conducted to assess whether [$^2H_5$]tryptophan was being converted to [$^2H_5$]IPyA in pea roots. As shown in Figure 5.6, no signal for [$^2H_5$]IPyA was found in those plants treated with [$^2H_5$]tryptophan, in comparison to those left untreated. However, this absence may be due to the instability of IPyA prior to derivatisation.

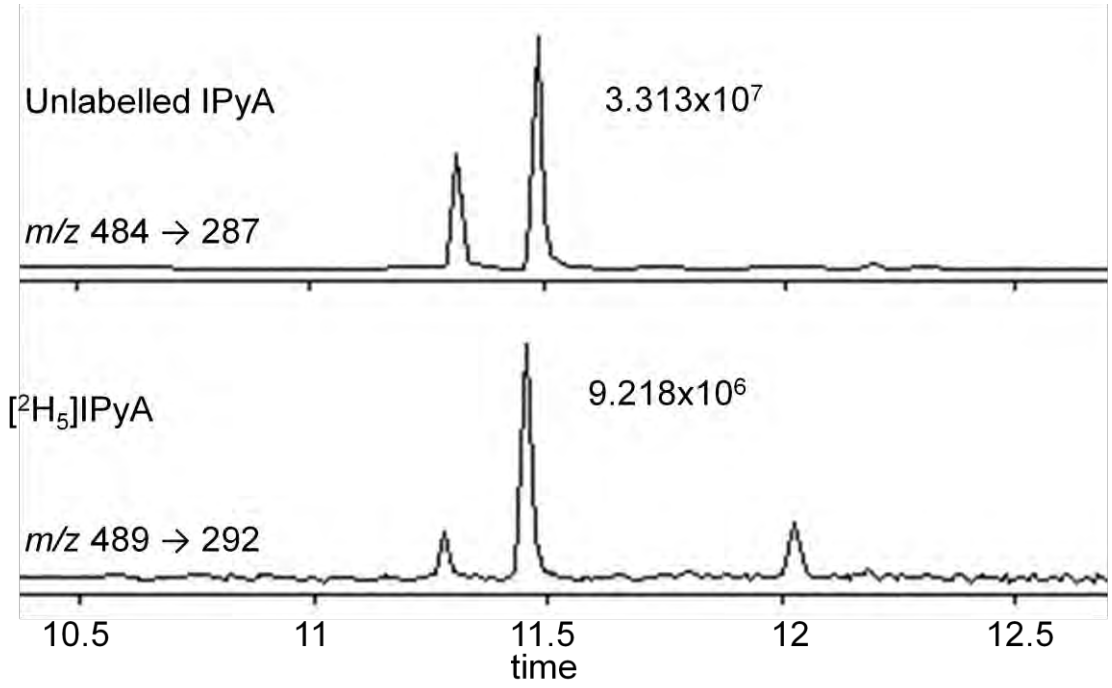

Figure 5.2. GC/MS/MS chromatograms from 500 ng synthetic IPyA (top) and 50 ng [$^2H_5$]IPyA (bottom): The peak areas were much lower than expected for the amount of material analysed.

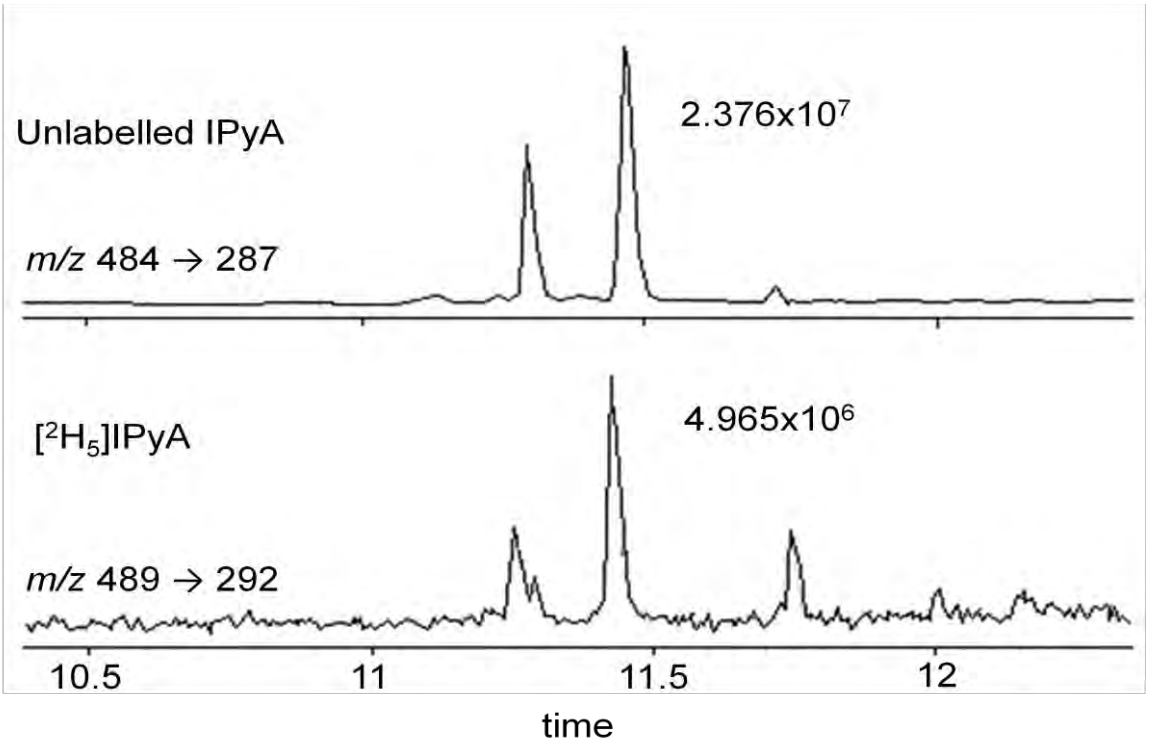

Figure 5.3. GC/MS/MS chromatograms from 500 ng synthetic IPyA (top) and 50 ng [$^2H_5$]IPyA (bottom), as shown in Figure 5.1., after one week. The signals were much lower than the previous week.

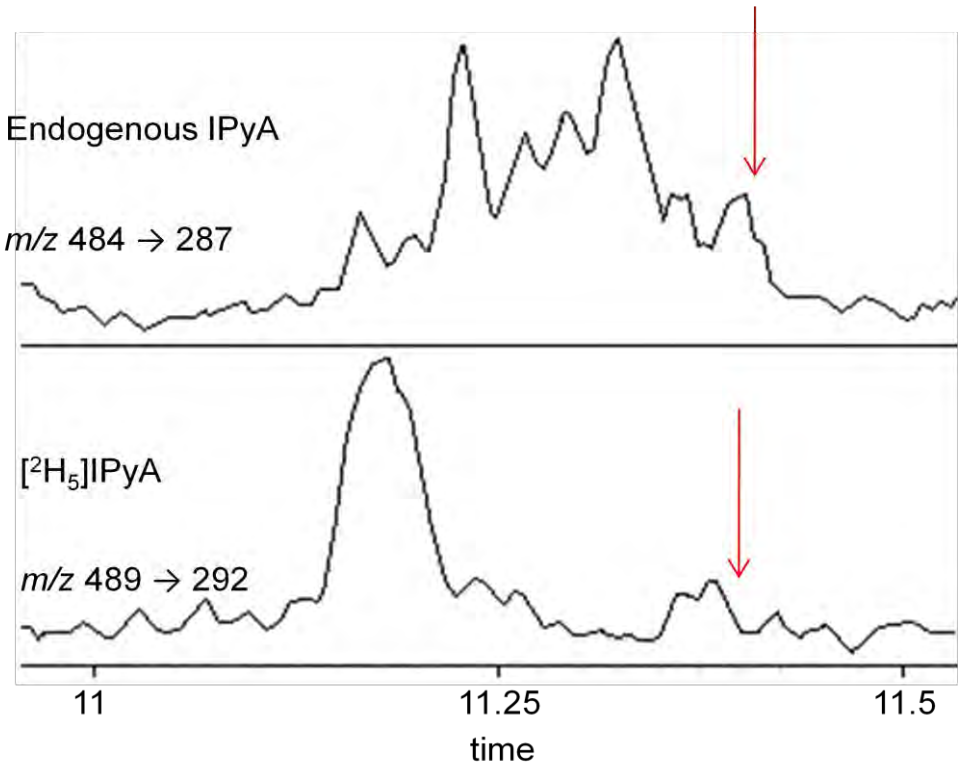

Figure 5.4. GC/MS/MS chromatograms from a pea shoot extract. The top channel (484 → 287) shows where IPyA was expected to elute (red arrow). The bottom channel (489 → 292) shows the expected retention time for [$^2H_5$]IPyA (red arrow). Despite the addition of 50 ng [$^2H_5$]IPyA internal standard, no clear peak that would be unequivocally identified as IPyA was evident in any run.

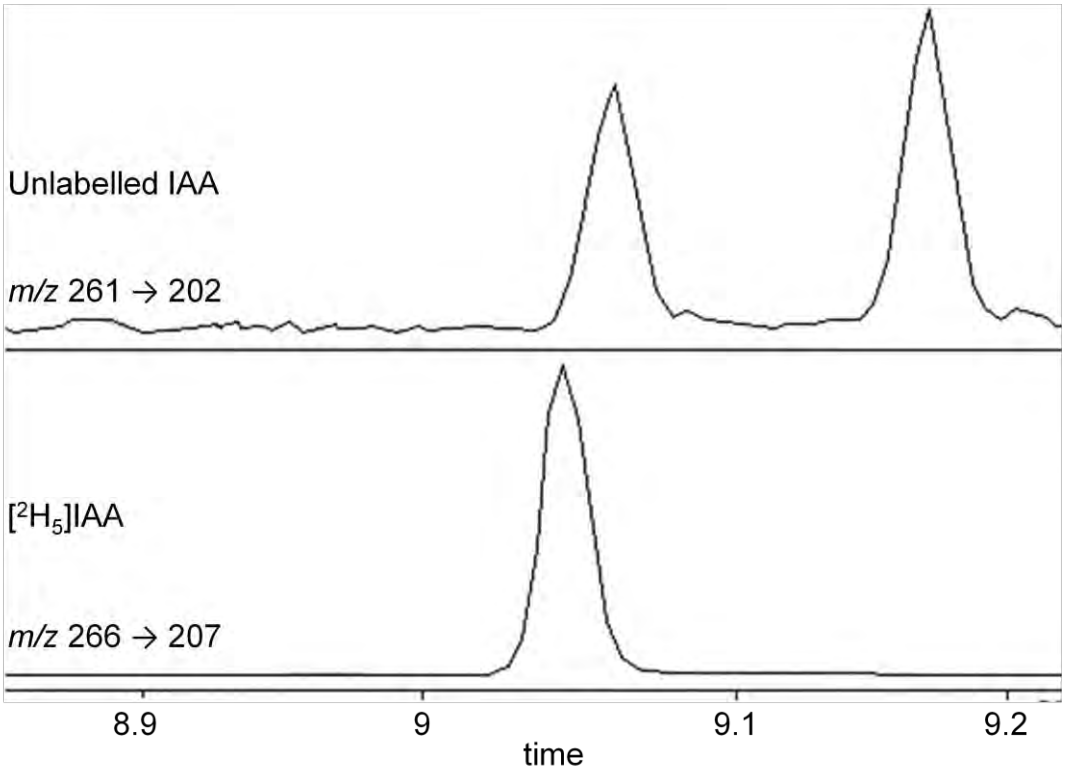

Figure 5.5. GC/MS/MS chromatograms from 500 ng synthetic IPyA and 50 ng [$^2H_5$]IPyA left for one week at 20˚C. The chromatograms show a peak eluting at the correct retention time, and in the correct channel (261 → 202) for IAA (top), as well as in the channel for [$^2H_5$]IAA (266 → 207; bottom).

Table 5.1. Analysis of the stability of the signal of synthetic IPyA after derivatisation, compared to the relative peak areas for [$^2H_2$]IEt, as analysed by GC/MS/MS. Two identical extracts (200 ng IPyA: 20 ng [$^2H_2$]IEt) were analysed for the relative peak area of IPyA compared to [$^2H_2$]IEt: Extract **A** was analysed by GC/MS/MS initially, and then after 72 and 96 hours, allowing a proportion of it to evaporate at room temperature. Extract **B** was analysed after being sealed at room temperature for 96 hours, and then after 10 days (again, allowing some evaporation). The peak areas for both [$^2H_2$]IEt and IPyA are shown, along with the ratio of IPyA to [$^2H_2$]IEt peak area. The transitions monitored for IPyA show a lower signal than the transitions monitored for [$^2H_2$]IEt – despite the addition of 200 ng of the former, and only 20 ng of the latter.

Extract	**Description (200ng IPyA: 20 ng [$^2H_2$]IEt)**	**[$^2H_2$]IEt peak area**	**IPyA peak area**	**Ratio IPyA to [$^2H_2$]IEt**
A	Initial analysis (day 1)	1.36×10^8	2.68×10^7	0.2
A	Analysed after 72 hrs (1/3 evaporated)	5.85×10^8	1.50×10^8	0.26
A	Analysed after 96 hrs (1/2 evaporated)	5.88×10^8	1.36×10^8	0.23
B	Analysed after 96 hrs (sealed)	6.68×10^8	2.08×10^8	0.31
B	Analysed after 10 days (3/4 evaporated)	7.02×10^8	2.49×10^8	0.35

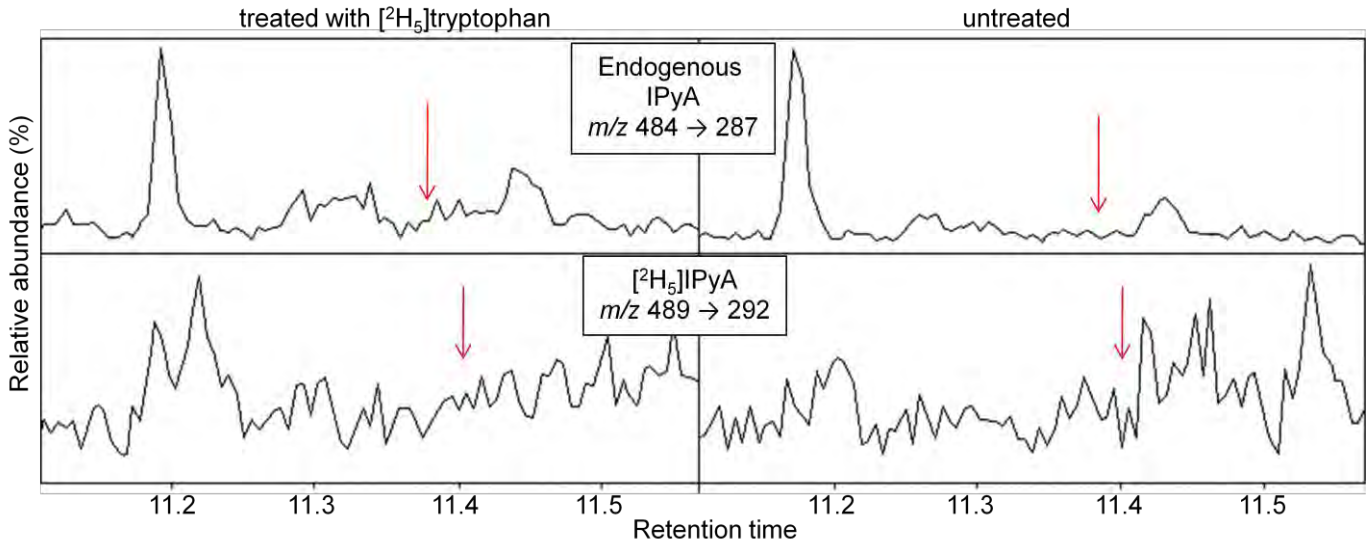

Figure 5.6. [$^2H_5$]tryptophan was not converted to [$^2H_5$]IPyA in pea roots. GC/MS/MS chromatograms showing no accumulation of label in IPyA from roots of whole peas incubated with [$^2H_5$]tryptophan (left), similar to untreated plants (right). The red arrows indicate where peaks were expected to appear.

5.4. Discussion

Due to its lability, IPyA is a difficult compound to isolate – as has been previously noted (Gibson et al., 1972b; Tam and Normanly, 1998). Synthetic IPyA was analysed herein by GC/MS/MS; however, it appears to degrade spontaneously to IAA in solution. Furthermore, [$^2H_5$]IPyA was not taken up successfully into plant roots, or degraded before uptake occurred. Degradation of IPyA to IAA was noted previously (Ernstsen et al., 1986), although these researchers did not use GC/MS/MS to verify the result. Because of this breakdown of IPyA to IAA through oxidation, it is not possible to successfully examine the metabolic fate of [$^2H_5$]IPyA *in vivo*.

Unfortunately, because of this discovery, previous studies come into question. The compound was not found, despite efforts to do so, in maize kernels (Srivastava, 1964), or pea seedlings (Gibson et al., 1972b; Schneider et al., 1985). It is possible that the reason for this lack of isolation is the degradation of IPyA to IAA, as opposed to actual absence (Nonhebel et al., 1993).

It appears that after derivatisation, IPyA is relatively stable. It was thought that IPyA would be too unstable to analyse by GC/MS/MS due to the high temperatures involved in the necessary derivatisation in preparation for analysis (Glassbrook et al., 2000), where other techniques such as LC/MS may be more appropriate because of the compounds lability (Wilbert et al., 1998). However, the results herein suggest that this derivatisation process actually stabilizes the compound, making its analysis possible. Tam and Normanly (1998) used GC/selected ion monitoring/MS to identify and quantify IPyA in *Arabidopsis* (3.85 to 13.08 ng g FM, varying with age of seedling), and also found that derivatisation was necessary for reliable quantification and stability of the compound. The current study was not successful in isolating IPyA within peas, however, was in agreement with the findings of Tam and Normanly (1998).

There was no evidence that tryptophan was converted to IPyA in pea roots. However, as mentioned previously, it is possible that the absence of IPyA may be due to the instability of this compound prior to derivatisation. It is possible that a pathway to IAA via IPyA is operative in peas. As shown in Chapter 4, tryptophan is metabolized *in vivo* to IAAld, IEt and IAA. These results not only suggest that the tryptamine pathway is active in pea, but also could implicate the IPyA pathway in IAA biosynthesis. Both IAAld and IEt are postulated to

be constituents of the IPyA pathway to IAA, and it is possible that the route through IPyA is also operative, as suggested for *Arabidopsis* (Stepanova et al., 2008; Tao et al., 2008). Without stable labelled forms, or inhibitors of the key compounds, it is difficult to be sure of specific biosynthetic routes in the plant. Therefore, the pathway may, in fact, be important for the production of IAA in vegetative pea tissue; however, without metabolism experiments it is very difficult to pinpoint its importance from a physico-chemical perspective.

These studies on IPyA, for both quantitation and metabolism purposes, were rendered difficult by the instability of this compound, which spontaneously degraded to IAA itself. Further study involving the stabilization of IPyA, particularly in relevance to metabolism studies, is needed in order to evaluate the relevance of the IPyA pathway to IAA biosynthesis.

CHAPTER 6

The Indole-3-Acetamide Pathway to IAA

6.1. Introduction

Until recently, the IAM pathway to IAA was generally accepted as the route bacteria used to produce the hormone: IAM has been implicated as an intermediate in the bacterial pathway for decades (Patten and Glick, 1996). However, recent evidence indicates the possible involvement of this precursor in higher plants. The pathway proceeds from tryptophan to IAM via tryptophan-2-monooxygenase, followed by the conversion of IAM to IAA by indoleacetamide hydrolase (Pollmann et al., 2006a; Figure 6.1). Numerous genes responsible for these conversions have been cloned from bacteria such as *Agrobacterium*, *Pseudomonas* and *Pantoea* (Spaepen et al., 2007).

Early reports of the presence of IAM in plants such as *Citris unshiu* (Igoshi et al., 1971), *Prunus jamasakura* (Saotome et al., 1993), and IAM hydrolase activity in *Triticum aestivum* (Fawcett et al., 1960), pea and rice (Kawaguchi et al., 1991; Kawaguchi et al., 1993) were generally overlooked due to problems with bacterial contaminations (Lehmann et al., 2010). However, the activity and presence of IAM has been reported in aseptically grown squash seedlings (Rajagopal et al., 1994), and an IAM hydrolase was identified and functionally characterised in tobacco (*NtAMI*; Nemoto et al., 2009). Furthermore, in *Arabidopsis*, levels of IAM are similar to IAA, and *Arabidopsis* amidohydrolase appears to convert IAM to IAA *in vitro* (Pollmann et al., 2002; Pollmann et al., 2003; Pollmann et al., 2006b).

Recently, IAM has been unequivocally isolated in *Arabidopsis*, maize, rice and tobacco by LC/MS (Sugawara et al., 2009). Furthermore, isotope dilution experiments have shown that the conversion of tryptophan to IAA *in vitro* by *Arabidopsis* protein extracts is affected by exogenous IAM, suggesting its involvement in IAA biosynthesis (Pollmann et al., 2009). In this same study, incorporation of label was shown to occur in IAM after tryptophan incubation *in vitro*, and subsequently in IAA, but this was not shown in whole *Arabidopsis* plants, and therefore may not be representative of an *in vivo* system.

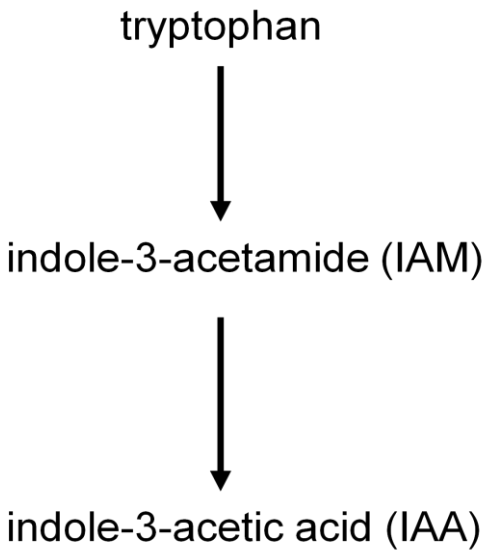

Figure 6.1. The IAM pathway to IAA proceeds from tryptophan to IAM by tryptophan-2-monooxygenase, and then from IAM directly to IAA by indoleacetamide hydrolase. Adapted from Woodward and Bartel (2005).

This evidence certainly points to a possible involvement of the IAM pathway to IAA in higher plants. However, the question remains as to whether the pathway is widespread, or restricted to certain families, and whether the pathway is operative within whole plant systems. Herein, the IAM pathway is investigated in pea: the development of techniques for IAM isolation by Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC/MS) is outlined; evidence is presented for the absence of IAM in pea despite its presence in corn, tobacco and *Arabidopsis*; and incubation studies are conducted using deuterium labelled tryptophan and IAM to target IAM and IAA, respectively.

6.2. Materials and Methods

6.2.1. Plant material

For identification and quantification studies, pea plants of line 107, or *bushy* mutant, heterozygote *BSHbsh* and wild type plants (as described in Chapter 7) were grown in glasshouse conditions as described in Chapter 2.1. In metabolism studies, pea seedlings (generally approximately 100 seeds) were grown as described (Chapter 2.1.2), and plants were incubated with either no substrate, or [$^2H_5$]tryptophan (10 μM), or in separate experiments, unlabelled IAM (10 μM; -Aldrich, St. Louis, MO, USA) or [$^2H_6$]IAM (5 μM; synthesised by Dr. Jason Smith, School of Chemistry, UTAS). All shoot and/or root material was then harvested and homogenized as described in Chapter 2.1.1. For each replicate, at least 6 plants were used for harvested material.

In separate experiments, corn and tobacco plants were grown for approximately seven weeks in the same glasshouse conditions (Chapter 2.1), and *Arabidopsis* for 34 days in a growth cabinet held at 20˚C with a 16 hour/day mixed light source of cool fluorescent white, and a humidity of 70%. Leaf and stem material (each replicate containing 4 plants) from tobacco, leaves and tassels from corn, as well as entire shoots from *Arabidopsis* seedlings, were harvested after these time periods, with harvesting and homogenization conditions the same as described for pea (Chapter 2.1.1). An internal standard of 10 ng/g [$^2H_2$]IEt, 10ng/g [$^{13}C_6$]IAA and/or 5ng/g [$^2H_6$]IAM internal standard was used for all quantification studies.

6.2.2. Extraction procedures

After this, aliquots of the harvested extracts for the analysis of IAM, IAAld, IEt and IAA were reduced under vacuum at 30°C to a small volume (1 to 4 mL) using a rotary evaporator. Extracts were purified with Sep-Pak C_{18} cartridges pre-conditioned with 100% methanol

followed by 0.4% acetic acid in dH_2O. Three 1 mL washes of 0.4% acetic acid were used to load the extract, which was washed with 1 mL 10% methanol in 0.4% acetic acid, and 12-15 mL 50% methanol in 0.4% acetic acid was used to elute the compound. This eluate was dried under vacuum, transferred using 100 µl of 1% acetic acid in dH_2O into an eppendorf tube, and centrifuged for 2 minutes at 13,000 rpm. 50 µl of the extracts were then transferred to auto-sampling vials for UPLC/MS analysis, as described below. For analysis involving synthetic standards, 100 ng IAM and 20 ng [$^2H_6$]IAM were taken up in 50 µl 1% acetic acid to auto-sampling vials for UPLC/MS analysis.

6.2.3. UPLC/MS analysis

Samples were analysed by UPLC/MS using a Waters Acquity H-series UPLC coupled to a Waters Xevo triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). A Waters Acquity UPLC BEH C_{18} column (2.1 x 100mm x 1.7 micron particles) was used. The solvents were 1% acetic acid in water (Solvent A) and acetonitrile (Solvent B). Outlined here are the LC and MS protocols for the analysis of samples throughout this chapter – for each chromatogram shown, the LC and MS program is listed in its subheading.

UPLC Program A: Initially 80% A: 20 % B, to 50% A : 50% B at 4.5 minutes, held for 30 seconds, followed by re-equilibration to starting conditions for 3 minutes. The column was held at 35°C, and the flow rate was 0.25 mL min.

UPLC Program B: Initially 99% A: 1% B, to 80% A: 20% B at 8 minutes, then to 40% A: 60% B at 12 minutes, followed by re-equilibration to starting conditions for 3 minutes. The flow rate was 0.35 mL min, and the column was at room temperature (~35°C).

UPLC Program C: As for program B except the column was held at approximately 25°C.

UPLC Program D: Initially 99% A: 1% B, to 75% A: 25% B at 10 minutes, followed by re-equilibration to starting conditions for 3 minutes. Flow rate was 0.35 mL min, and the column was at room temperature (~ 23 °C).

UPLC Program E: As for program A but flow rate was 0.35 mL/min.

The mass spectrometer was operated in positive ion electrospray mode with needle voltage of 2.5 KV, and selected reaction monitoring was used to detect all analytes. Data was analysed using MassLynx software. The MS methods were as follows:

MS program A: The program ran for five minutes: but IAM and [$^2H_6$]IAM eluted between 0 and 2.75 minutes. The channel monitored for IAM was *m/z* 175.1 to 130.05, and for [$^2H_6$]IAM, *m/z* 181.1 to 135.05 and 181.1 to 134.05. The cone voltage was 24V, collision energy was 15V, and dwell time was 161 ms per channel. The ion source temperature was 150°C, the desolvation gas was nitrogen at 950 L per hr, the cone gas flow was 50 L per hr and the desolvation temperature was 300°C.

MS program B: This program ran for 10 minutes – and involved two functions. Function 1 monitored *m/z* 175.10 to 86.05 and *m/z* 175.10 to 88.05 (both for the IAM impurity), as well as for the true IAM transition *m/z* 175.10 to 130.05, and [$^2H_6$]IAM transitions *m/z* 181.1 to 136.05 and 181.1 to 135. The cone voltage was 24V, collision energy 15V. This same program also monitored the IAA *m/z* 176.05 to 130.05, and [$^{13}C_6$]IAA *m/z* 182.1 to 136.1, with cone voltage at 18V, and collision energy at 18V. The dwell time for all channels was 80ms. The second function of this program was a ScanWave of the daughter ions of *m/z* 175.1, scanning from *m/z* 40 to 185 in 30 ms, with the collision energy at 15V. Other ion source conditions were as for MS method A. The ion source temperature was 130°C, the desolvation gas was nitrogen at 950 L per hr, the cone gas flow was 100 L per hr and the desolvation temperature was 450°C.

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MS program C: This program involved two functions, over 8 minutes. Function 1, from 1.3 to 2 minutes, monitored the transitions for IAM (*m/z* 175.1 to 130.05), [$^2H_5$]IAM (*m/z* 180.1 to 135.1), and [$^2H_6$]IAM (*m/z* 181.1 to 136.1). The cone voltage was 24V, the collision energy 15V, with a dwell time of 128 ms. The second function, from 2 to 8 minutes, monitored IEt (*m/z* 162.1 to 144.05), [$^2H_5$]IEt (*m/z* 167.1 to 149.05), IAA (*m/z* 176.15 to 130.1), [$^2H_5$]IAA (*m/z* 181.15 to 135.1), and [$^2H_6$]IAM (*m/z* 182.15 to 136.1). The cone voltage was 18V, the collision energy 18V, with a dwell time of 52 ms.

Accurate mass measurements were performed by direct infusion of a pea extract portion collected over the suspected 'IAM' retention time of 6.5 to 6.8 minutes by UPLC/MS, into an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Scoresby, VIC) through a nano

electrospray capillary (Proxeon biosystems, Scoresby, VIC). Mass spectra were acquired in positive ion mode at a resolution of 30,000 and over a scan range of *m*/*z* 50-1000. The detection limit for IAM was 300 pg g[FW]$^{-1}$, and for IAA was 90 pg g[FW]$^{-1}$.

6.3. Results

6.3.1. UPLC/MS method development for the isolation of IAM: an investigation of pea root and shoot extracts, as well as Arabidopsis*, corn and tobacco extracts.*

An initial examination of pea root and shoot extracts exhibited a peak eluting just after [$^{2}H_{6}$]IAM, corresponding to the correct transition (175.1 → 130.05) for endogenous IAM (Figure 6.2). However, an investigation into the retention time expected for unlabelled IAM, in comparison to [$^{2}H_{6}$]IAM, showed that the initial 'IAM' found within pea extracts did not behave as expected. It appeared that the retention time for this plant 'IAM' was 0.1 minutes later than the [$^{2}H_{6}$]IAM internal standard (Figure 6.2), and the synthetic known IAM eluted 0.06 minutes after the [$^{2}H_{6}$]IAM internal standard (Figure 6.3). This result made it unclear as to whether the 'IAM' peak that was being investigated (Figure 6.2) was true IAM, or an unknown compound: i.e. whether IAM was present or absent in peas.

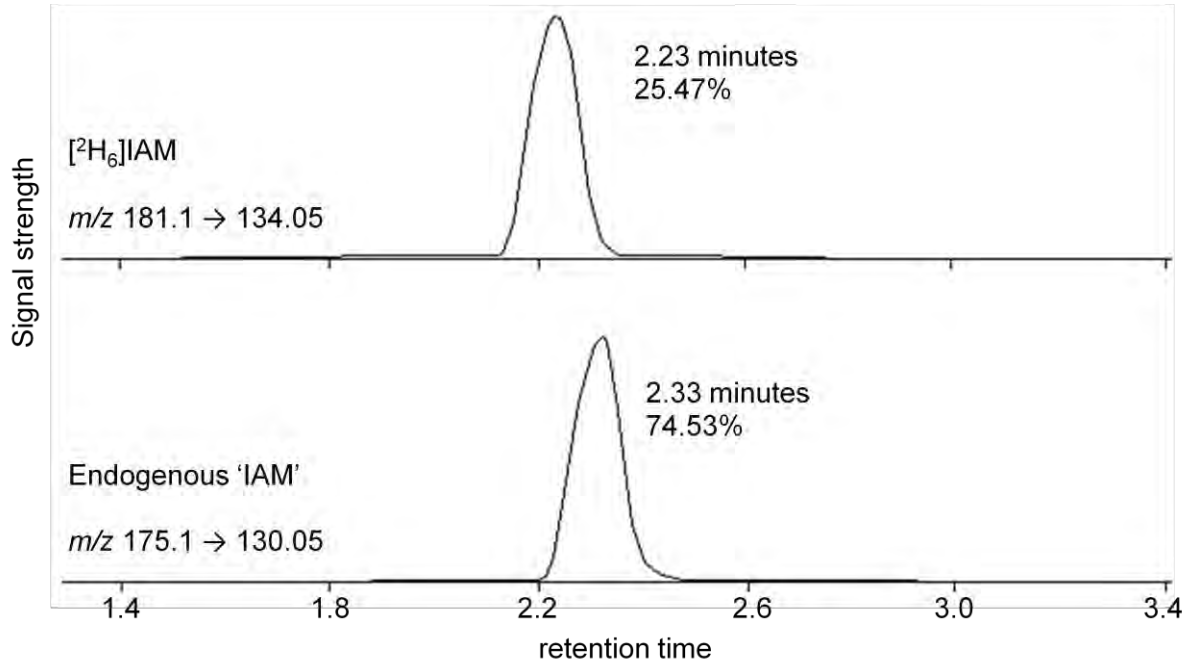

Figure 6.2. Chromatograms of putative endogenous 'IAM' isolated in pea shoot tissue, as compared to [$^2H_6$]IAM internal standard by UPLC/MS. The top peak (*m/z* 181.1→134.05) is the genuine internal standard, [$^2H_6$]IAM (eluting at 2.23 minutes). The bottom peak (*m/z* 175.1→130.05) is putative endogenous 'IAM' (eluting at 2.33 minutes). This shows a retention time shift of 0.1 minutes for unlabelled 'IAM' compared to the [$^2H_6$]- labeled version of the compound. The method for producing this chromatogram was LC program A and MS program A.

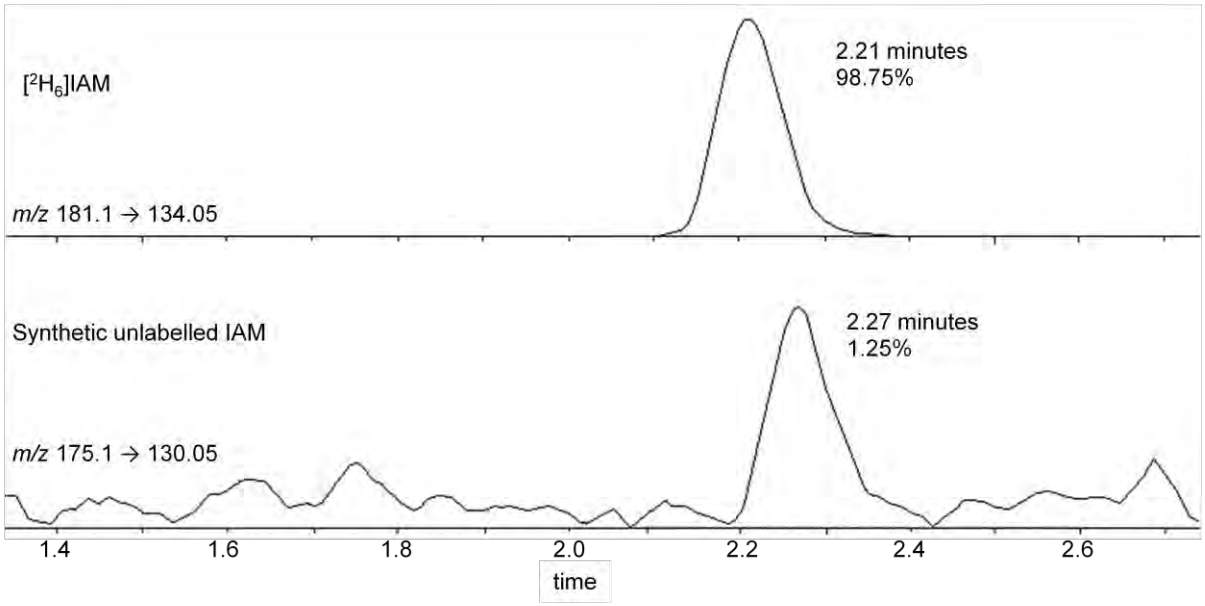

Figure 6.3. Chromatograms of 100 ng synthetic standard IAM co-eluting with 20 ng [$^2H_6$]IAM by UPLC/MS. The top peak (*m/z* 181.1→134.05) is genuine [$^2H_6$]IAM (eluting at 2.21 minutes); the bottom peak (*m/z* 175.1→130.05) synthetic unlabelled IAM (eluting at 2.27). This shows an expected retention time shift of 0.06 minutes for unlabelled IAM compared to the [$^2H_6$]- labeled version of the compound. The method for producing this chromatogram was LC program A and MS program A.

The initial studies involved an UPLC/MS column temperature of approximately 35ºC, and a fast program that eluted IAM within the range of 2.2-2.4 minutes. However, upon lengthening the program, plant 'IAM' appeared to shift to a retention time approximately 0.02 minutes before that of the [$^2H_6$]IAM internal standard (Figure 6.4A). Furthermore, lowering the temperature program to approximately 25ºC made the retention time of 'IAM' in pea extracts much shorter – it eluted 0.27 minutes before the [$^2H_6$]IAM internal standard (Figure 6.4B). Therefore, the compound co-eluting with [$^2H_6$]IAM, which was initially presumed to be IAM, was not, in fact, true IAM. This was confirmed using nano electrospray accurate mass detection (Figure 6.5): no peak for unlabelled IAM was shown at the correct mass (175.0886) for the putative 'IAM' fraction from pea tissue collected during UPLC/MS analysis. Therefore, IAM was not detected by UPLC/MS analysis at the detection limit of 300 pg g[FW]$^{-1}$.

In a separate experiment, pea *bushy* mutants, heterozygote *BSHbsh*, and wild type plants were analysed for the presence of IAM in apical tissue. A previous study had found that some IAA precursor levels may be higher within the mutant (Quittenden, 2005). If IAM were involved in IAA synthesis, it was possible that, if present, the levels of IAM would also be higher in the mutant. However, there was no peak detected at the appropriate retention time for endogenous IAM in any pea extracts further examined, *bushy* mutant, or wild type: that is, IAM is not an endogenous constituent of pea shoots or roots, or at least it may be present at very low levels, well below the detection limits available (as shown for *bushy* pea mutant shoots in Figure 6.6).

In separate experiments IAM was isolated, using the technique developed above, in *Arabidopsis*, corn, and tobacco plant extracts. Small amounts of the compound previously thought to be 'IAM' in peas was also identified in these species. The level of IAM in corn tassels (2.3 ng g[FW]$^{-1}$) was noticeably higher than the level in tobacco stem and leaf extracts (0.21 ng g[FW]$^{-1}$), or corn leaves (0.01 ng g[FW]$^{-1}$). However, the signal for IAM in these species was considerably lower than the 'IAM' impurity also found in pea. Conversely, in *Arabidopsis*, the signal for IAM was much greater than that of the impurity, as monitored by the transition *m/z* 175.1-88.05 that diagnostically shows a peak for the daughter ion of the impurity, but not for IAM (Figure 6.7). The detection of IAM in these species, and not in pea, is further confirmation that the compound is absent: or, if present, at very low levels.

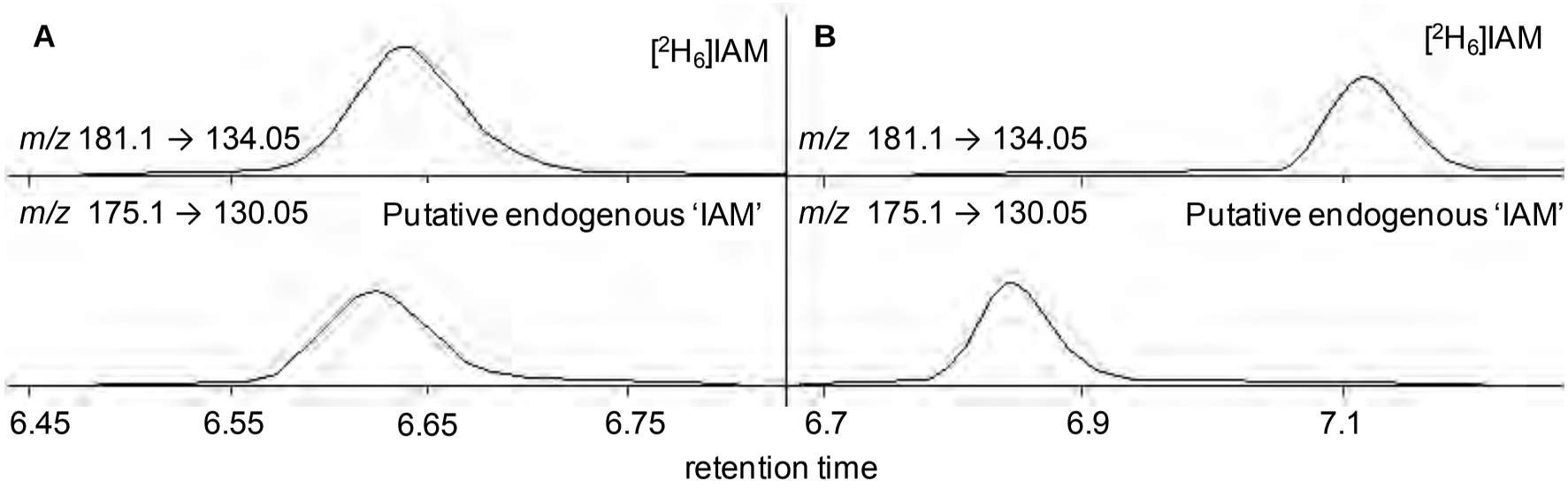

Figure 6.4. Chromatograms of pea root tissue subject to UPLC/MS analysis using two different temperature programs, and a slower gradient than Figures 6.1-2. **A** shows genuine [$^2H_6$]IAM (top) and putative endogenous 'IAM' (bottom) virtually co-eluting (IAM elutes 0.02 minutes before [$^2H_6$]IAM) at 35ºC. **B** shows genuine [$^2H_6$]IAM (top) and the putative 'IAM' peak not co-eluting, with the endogenous peak shifting to 0.27 minutes earlier at 25˚C, indicating that the compound is not IAM. The method for producing these chromatograms were LC program B (for the 35˚C column depicted in **A**), and LC program C (for the 25˚C column depicted in **B**), with both chromatograms produced by using MS program A.

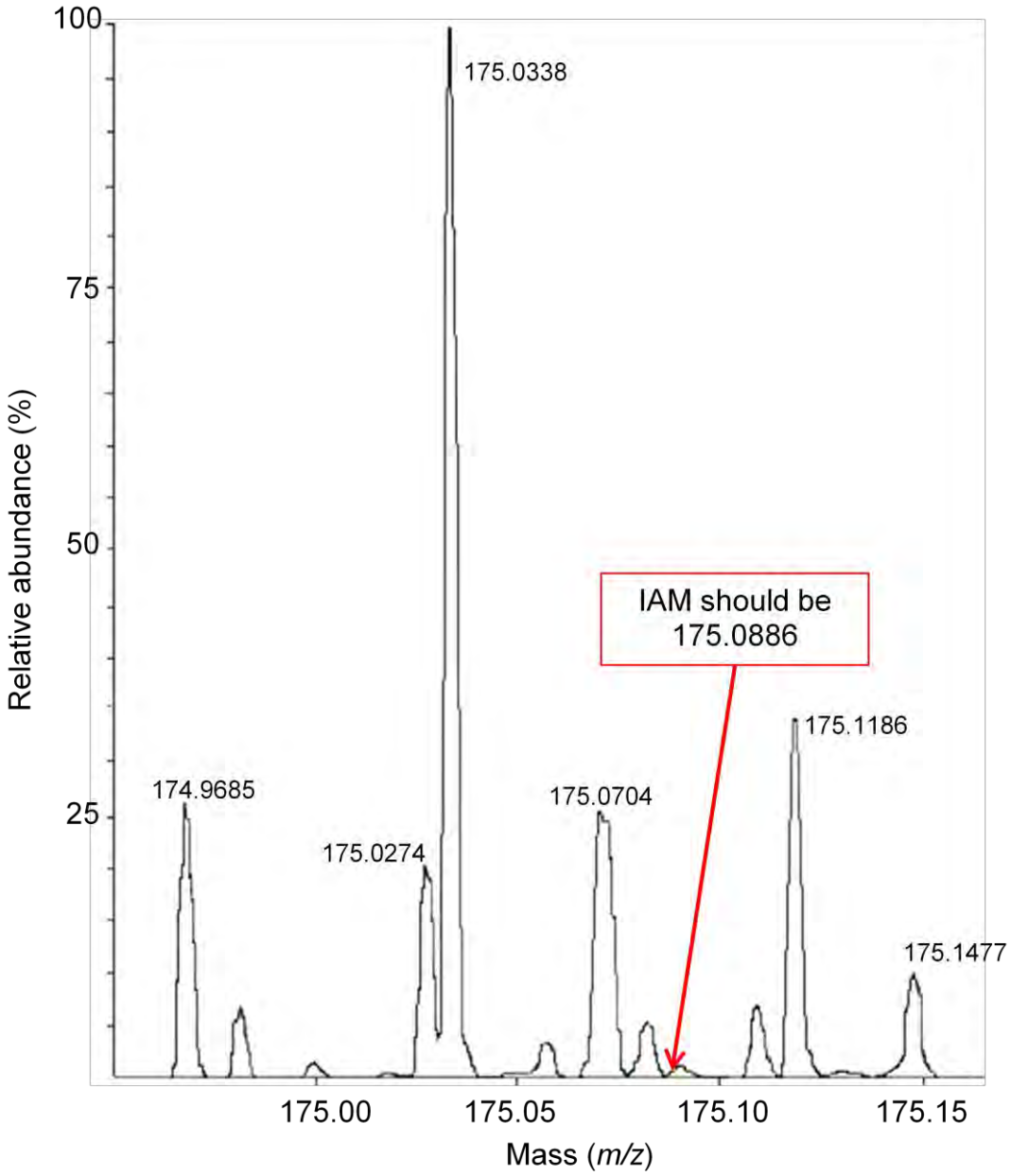

Figure 6.5. Chromatogram from nano electrospray accurate mass detection, showing masses detected in the putative 'IAM' fraction collected from pea tissue during UPLC/MS analysis. The most abundant peak is of the mass *m/z* 175.0338 does not match the expected mass for IAM of *m/z* 175.0886 (red): no peak was found for the correct IAM mass.

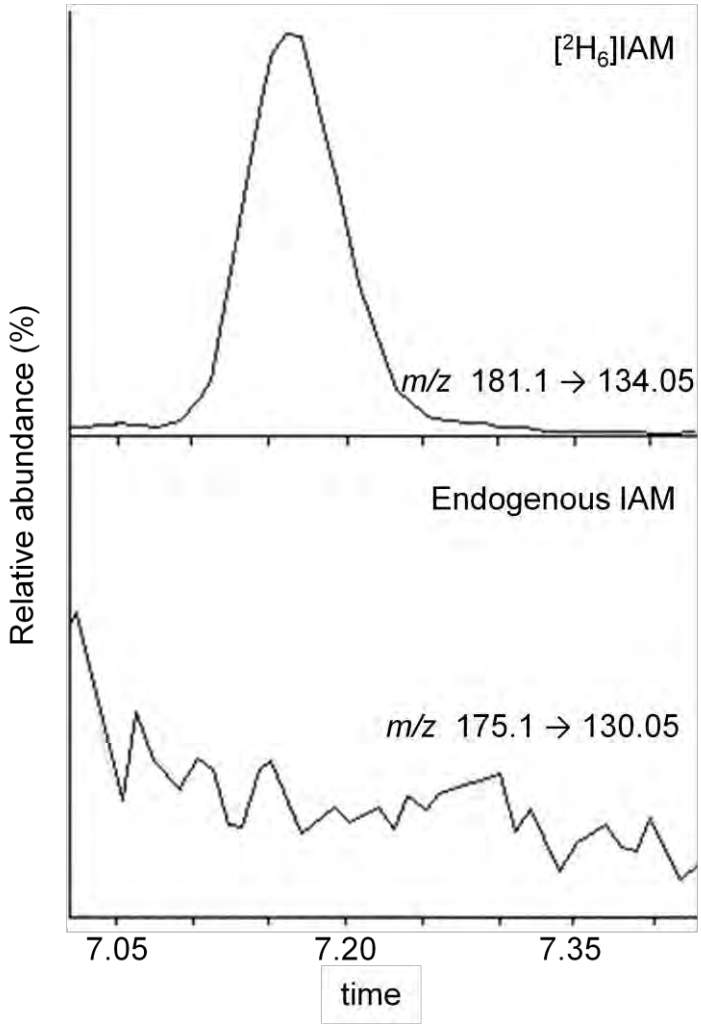

Figure 6.6. Chromatograms from UPLC/MS analysis of *bushy* pea mutant tissue at a column temperature of 25ºC, showing genuine internal standard [$^2H_6$]IAM (top) and no peak at the expected retention time for endogenous IAM (bottom). That is, IAM is not present in pea tissue. The method for producing this chromatogram was LC program C and MS program B.

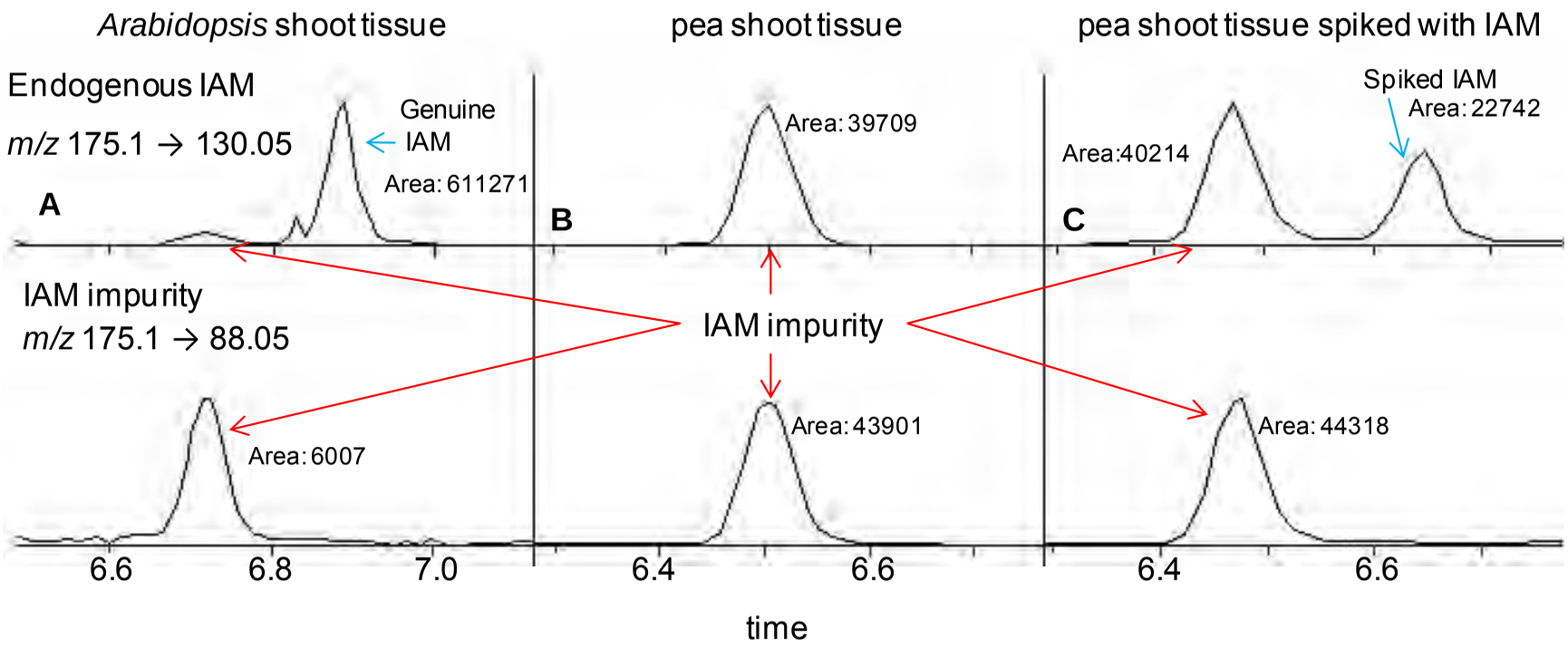

Figure 6.7. Chromatograms of *Arabidopsis* and pea root tissue subject to UPLC/MS analysis at 25˚ C. **A** shows *Arabidopsis* shoot tissue with genuine IAM (top; blue arrow) and a small peak co-eluting with IAM, the IAM impurity found previously in pea (bottom). **B** shows the IAM impurity co-eluting in pea tissue, with no peak for actual endogenous IAM. **C** shows pea shoot tissue as shown in B, but spiked with unlabelled IAM (blue arrow). As shown, the peak in **B** and **C** (marked with red arrows) is the same impurity found in *Arabidopsis* – not genuine IAM at all. The pea tissue, unlike the *Arabidopsis* tissue, does not contain endogenous IAM. The method for producing these chromatograms was LC program D and MS program B.

6.3.2. Metabolism of [$^2H_5$]tryptophan to [$^2H_5$]IAM

After incubation of pea roots with [$^2H_5$]tryptophan, there was no peak detected at the correct retention time for [$^2H_5$]IAM (Figure 6.8), even though [$^2H_5$]tryptophan was found in the roots: i.e. the substrate was taken up. That is, tryptophan was not converted to IAM in pea roots.

6.3.3. Metabolism of [$^2H_6$]IAM and unlabelled IAM to IAA

Although IAM was not detected in pea extracts, it was possible that the enzymatic machinery needed for the production of IAA from IAM was present and that IAM does not accumulate to a detectable level. Therefore, aseptic roots from whole pea seedlings were incubated with [$^2H_6$]IAM. No deuterium incorporation was seen within the IAA pool in comparison to the untreated roots after this incubation (Figure 6.9). That is, no conversion of [$^2H_6$]IAM to the appropriately labeled IAA was found. The roots were analysed for the presence of [$^2H_6$]IAM itself, and the compound was, indeed, taken up.

It appeared as though IAM was both not present in peas, and peas were not able to produce, or metabolise the compound. However, one interesting finding was that the levels of IAA found in pea roots after incubation with [$^2H_6$]IAM were dramatically decreased (Figure 6.10). The experiment was repeated using unlabelled IAM as a substrate, but this time there was no discernable decrease in IAA levels (Figure 6.11). If anything, a slight (but not significant) increase in both IAA and IAAld was found within those seedlings incubated with unlabelled IAM (Figure 6.12), and a decrease in IEt levels (Figure 6.13).

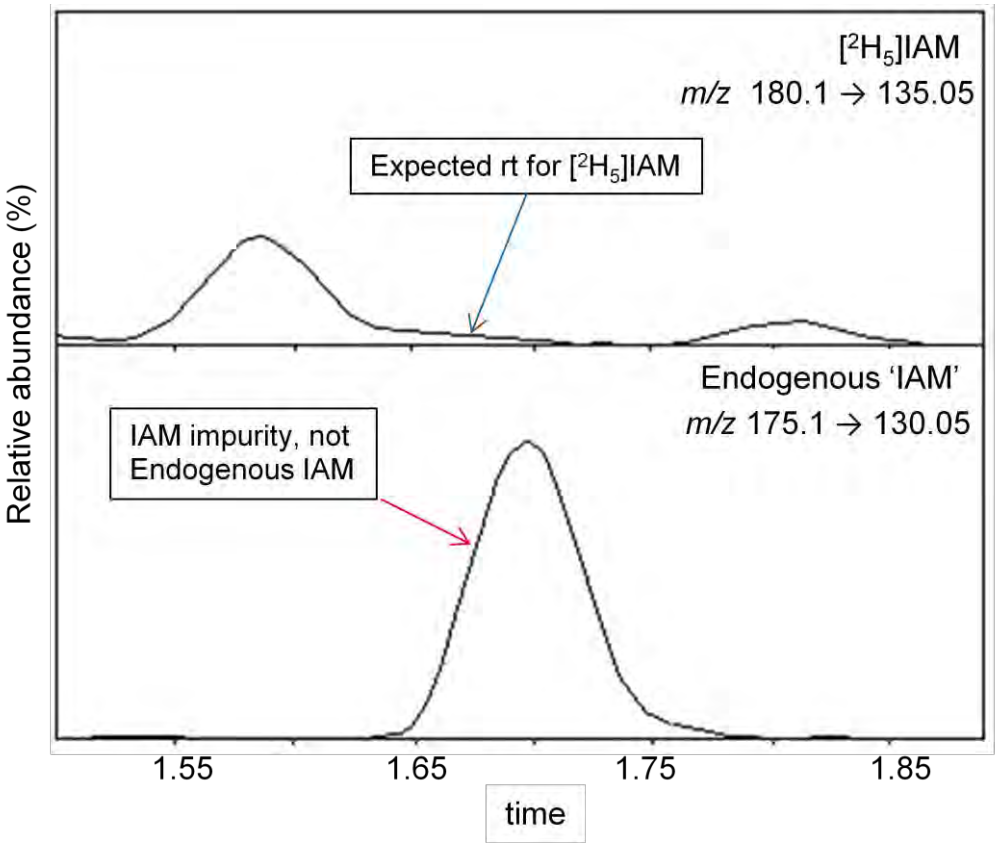

Figure 6.8. Chromatograms from UPLC/MS analysis at a temperature of 35˚C and a slow program, of pea root tissue incubated with [$^2H_5$]tryptophan. The peak in the endogenous channel (*m/z* 175.1→130.05; red arrow) is not IAM, but the compound that should co-elute with [$^2H_5$]IAM. No peak is shown in the [$^2H_5$]IAM channel (*m/z* 180.1→135.05; blue arrow), and therefore [$^2H_5$]tryptophan was not converted to [$^2H_5$]IAM. The method for producing this chromatogram was LC program E and MS program C.

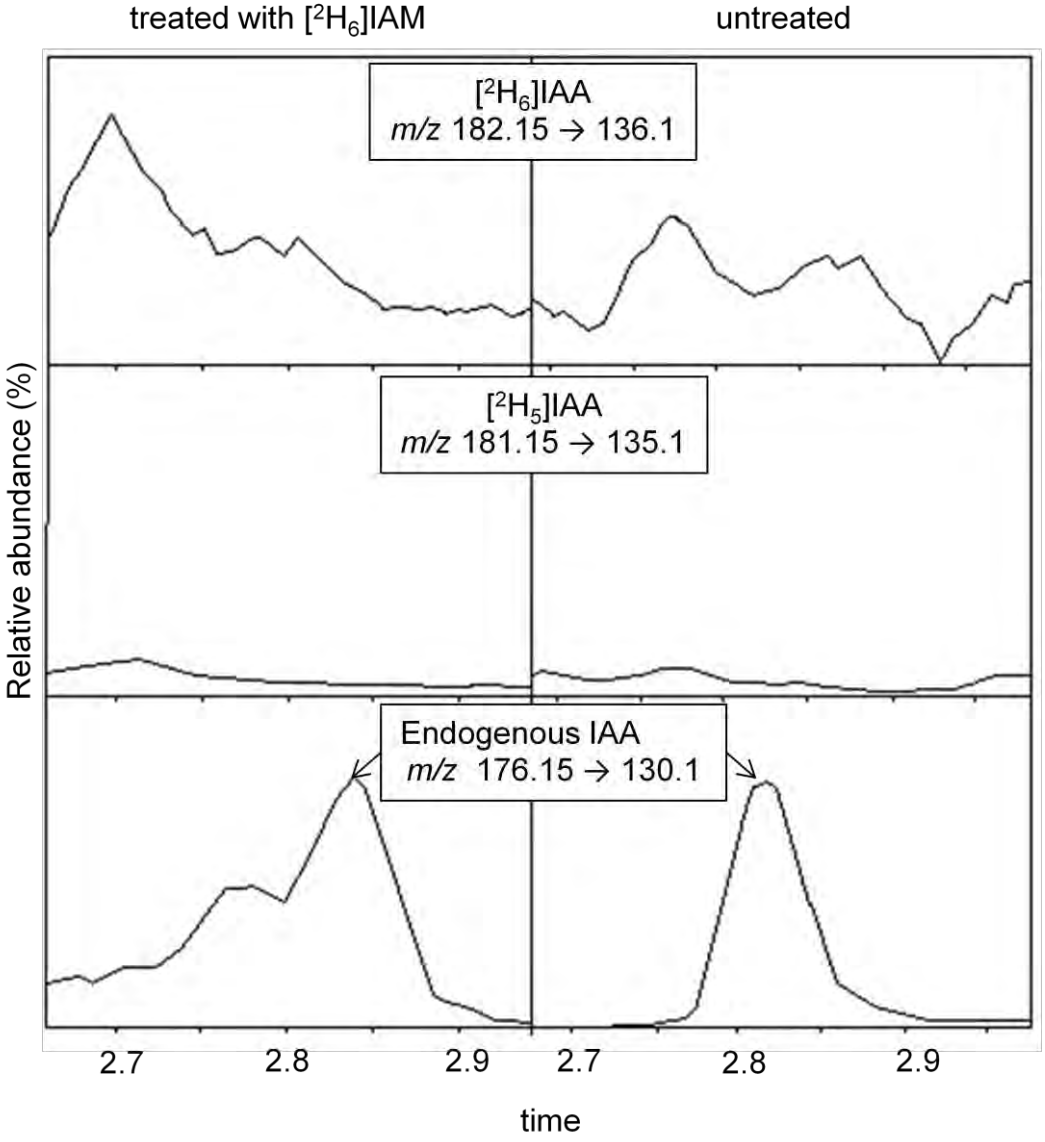

Figure 6.9. UPLC/MS chromatograms showing no conversion of [$^2H_6$]IAM to [$^2H_5$]IAA in pea roots on a slow program at 35˚C. No presence of a peak in either the [$^2H_6$]- (top) or [$^2H_5$]- (middle) channels for IAA were seen after treating pea roots with [$^2H_6$]IAM (left), in comparison to the controls (right). Endogenous IAA present in the roots is shown in channel *m/z* 176.15→130.1. There was a much lower signal for IAA on treatment with [$^2H_6$]IAM. The method for producing this chromatogram was LC program E and MS program C.

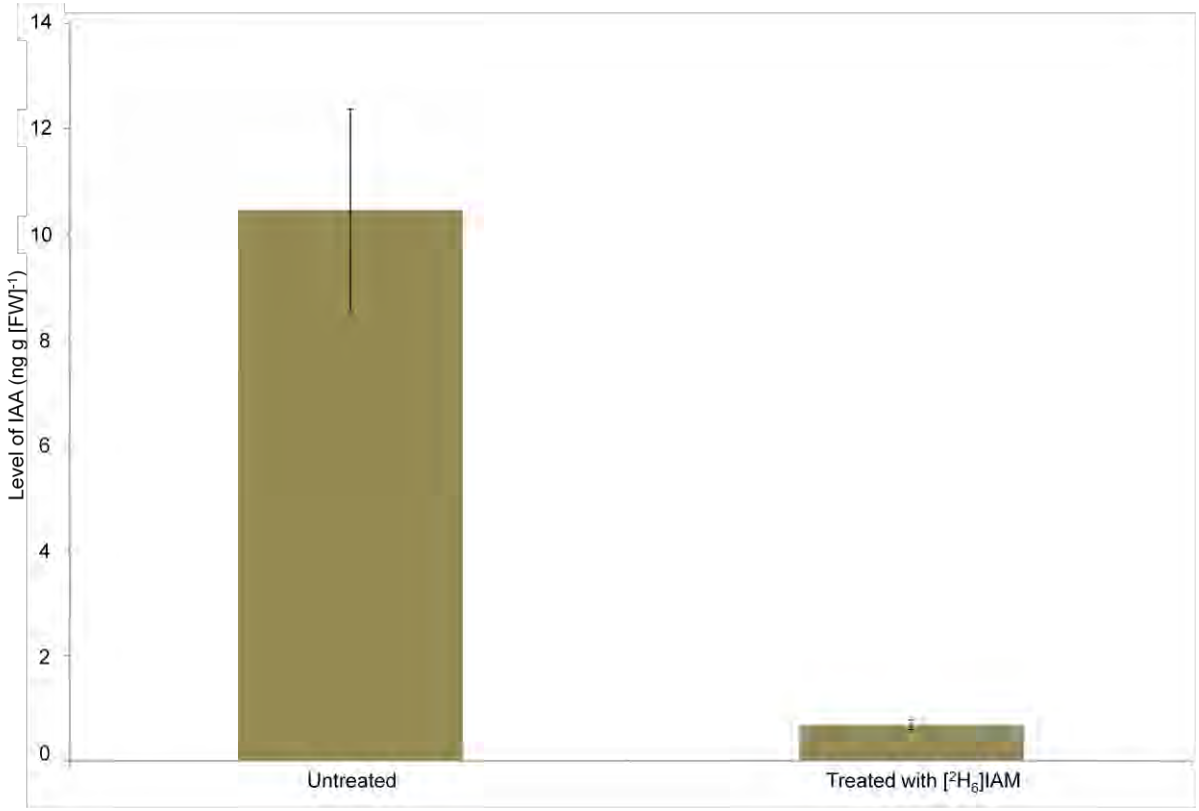

Figure 6.10. The level of IAA (ng g $[FW]^{-1}$) in pea roots treated with $[^2H_6]$IAM, and those left untreated, as analysed by UPLC/MS. Shown are the means ± standard error (n=2). This is the same experiment as depicted in Figure 6.9. The method for producing this data was LC program E and MS program C.

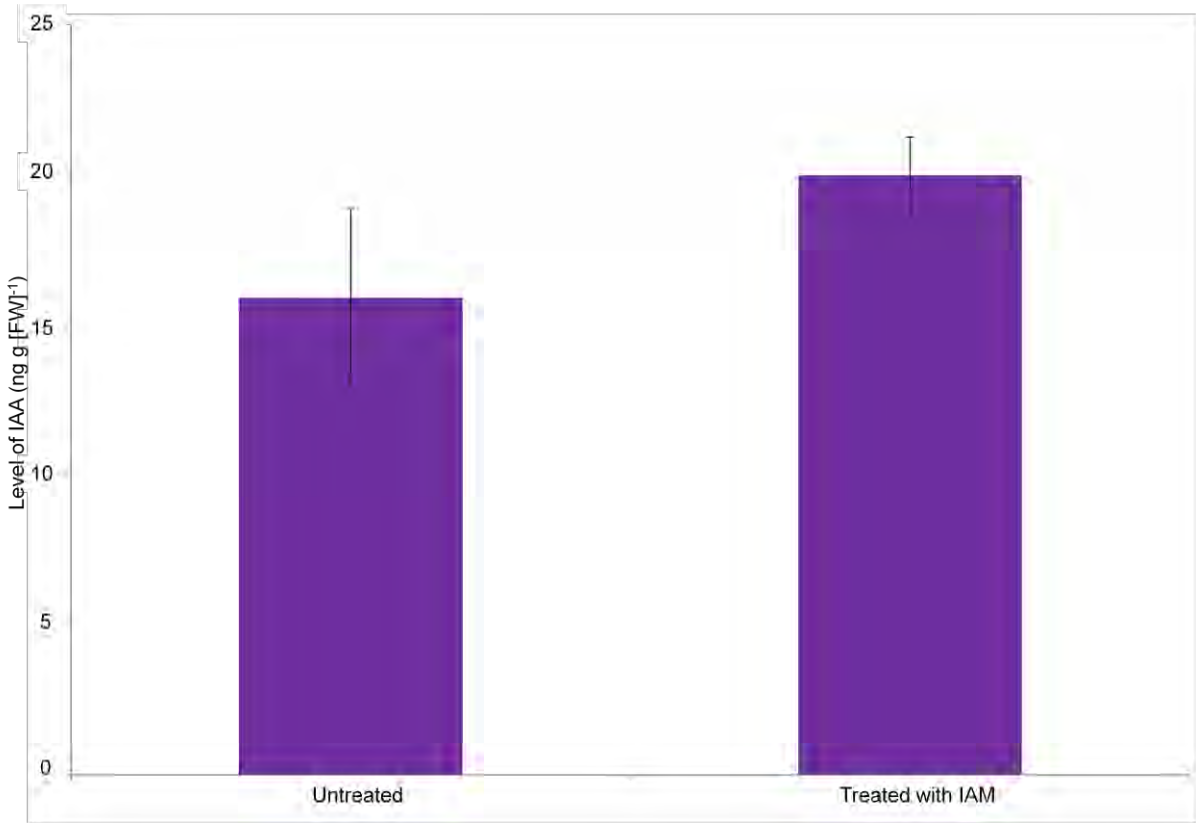

Figure 6.11. The level of IAA (ng g $[FW]^{-1}$) in pea roots treated with unlabelled IAM, and those left untreated, as analysed by UPLC/MS. Shown are the means ± standard error (n=3). The method for producing this data was LC program E and MS program C.

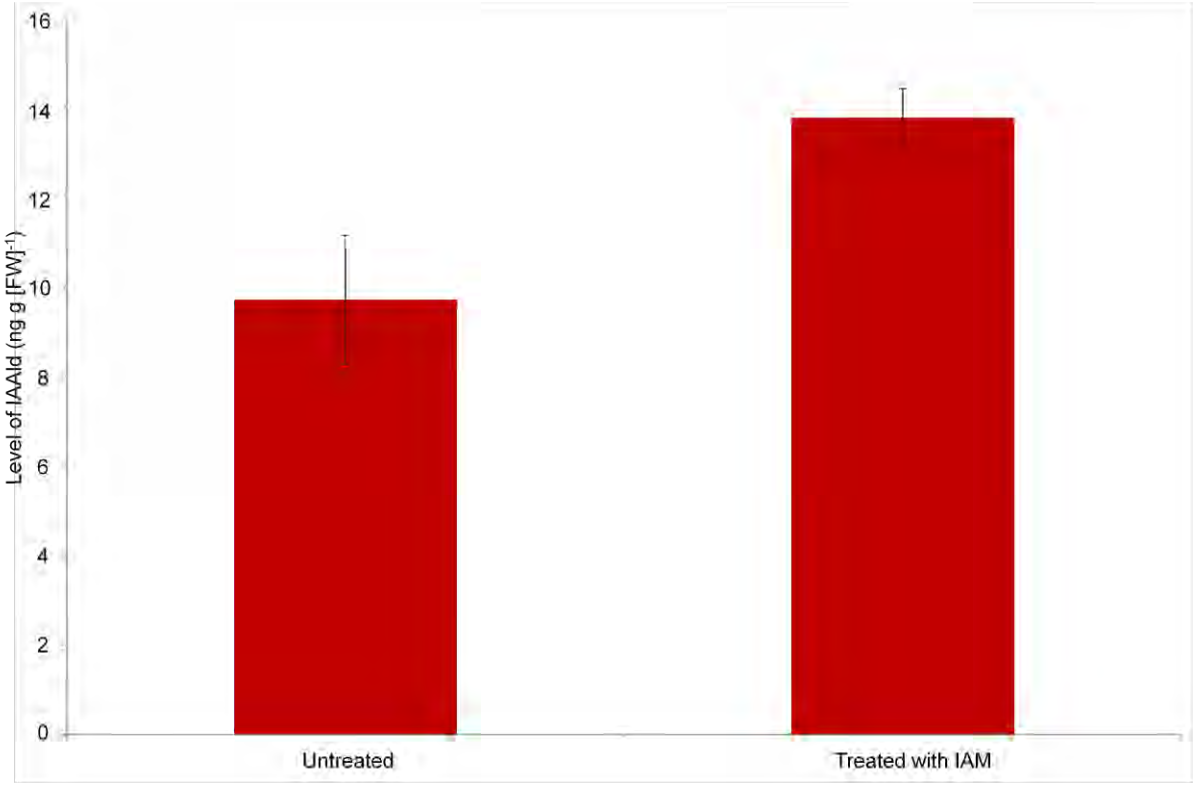

Figure 6.12. The level of IAAld (ng g $[FW]^{-1}$) in pea roots treated with unlabelled IAM, and those left untreated, as analysed by UPLC/MS. Shown are the means ± standard error (n=3). The method for producing this data was LC program E and MS program C.

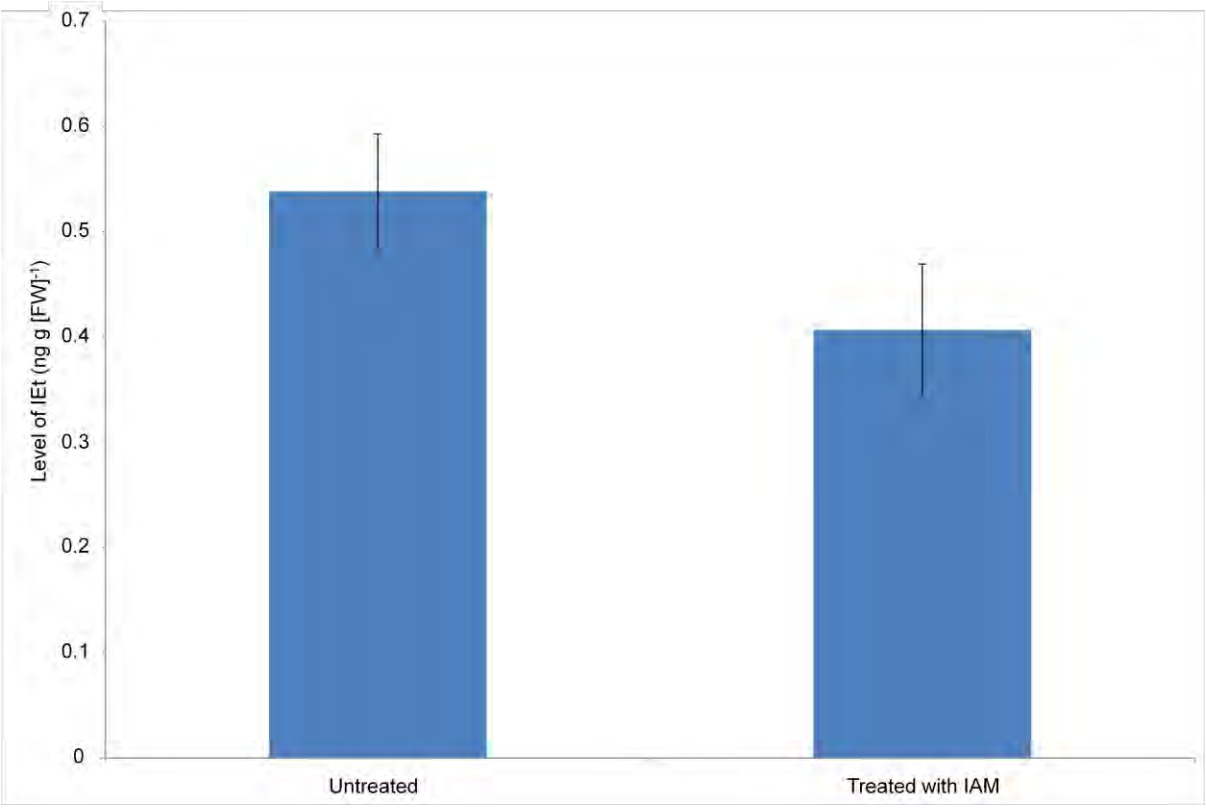

Figure 6.13. The level of IEt (ng g [FW]$^{-1}$) in pea roots treated with unlabelled IAM, and those left untreated, as analysed by UPLC/MS. Shown are the means ± standard error (n=3). The method for producing this data was LC program E and MS program C.

6.4. Discussion

Based on evidence from both quantification and metabolism studies, it appears that the IAM pathway to IAA does not operate in pea roots. IAM is not present in pea shoots or roots (or at least is at a very low level), despite being present in *Arabidopsis*, corn and tobacco tissue. Furthermore, it appears as though the [$^2H_6$]- form of the compound is not being converted to IAA in pea roots, and nor is [$^2H_5$]tryptophan being converted to [$^2H_5$]IAM.

The *bushy* pea mutant was used here as a diagnostic tool (as further explained in Chapter 7). Previous preliminary research found that the level of some IAA precursors (such as tryptamine) were higher in the mutant (Quittenden, 2005). Therefore, if IAM were involved with IAA biosynthesis, it was possible that the compound would be produced at a higher endogenous level within the mutant, if it were being produced at all. This was not the case in this circumstance: no IAM was detected in the mutant, thus further strengthening the conclusion that the compound is not present in vegetative pea tissue.

As outlined in the results, another compound eluted at a similar retention time to genuine IAM, as confirmed by studies using both synthetic material and those species mentioned above that genuinely contain IAM endogenously (*Arabidopsis*, corn and tobacco, as described previously; Pollmann et al., 2006b; Nemoto et al., 2009; Sugawara et al., 2009). This problem of co-elution highlights the need for stringent analysis techniques for the identification of hormones and their precursors within plant tissue (Kojima et al., 2009). Otherwise, the co-eluting peak may have been misinterpreted as genuine IAM – an assumption that was wrong.

Deuterated IAM was not converted to IAA within pea roots. Conversely, it appears as though IAA levels exhibit a dramatic decrease on the introduction of [$^2H_6$]IAM (10μM) to the *in vivo* plant root system. This is not so when pea roots are incubated with unlabelled IAM – where IAA levels are not significantly different in untreated and treated tissue. An explanation for this is not forthcoming; however, it is possible that there is another compound present in the [$^2H_6$]IAM used for incubation studies that acted as an inhibitor to IAA (such as IAA-methyl ester). Further investigation into this phenomenon is required to eliminate this possibility.

These findings are presented at a time when the IAM pathway is becoming a ‘hot topic’ in IAA biosynthesis. Other researchers have recently produced evidence that IAM is, in fact,

involved in IAA biosynthesis in higher plant species such as *Arabidopsis* (Pollmann et al., 2006b; Sugawara et al., 2009) and tobacco (Nemoto et al., 2009). The current evidence that the compound is not involved in pea roots tends to highlight that the IAM pathway to IAA may be restricted to certain plant families. Furthermore, the difficulty in isolating the compound using UPLC/MS due to the interference of another, unrelated, compound, indicates that caution is required in IAM identification and analysis.

CHAPTER 7

The *bushy* Mutant: A Key Tool for the Study of IAA Biosynthesis

7.1. Introduction

Mutants with disrupted hormone biosynthesis or metabolism pathways are key tools in the elucidation of developmental processes at work in plants. However, relatively few mutants with substantially reduced IAA levels are available, possibly due to inviability. More frequently, IAA overproduction mutants have been characterised, such as *Arabidopsis sur1* (Boerjan et al., 1995) and *sur2* (Barlier et al., 2000). In contrast, *bushy* is a dominant pea mutant with markedly reduced IAA levels, and a short, branching phenotype indicative of IAA reduction (Figure 7.1; Symons et al., 1999). It has been shown previously that *bushy* has lowered free IAA levels, and somewhat higher conjugated IAA levels, with [^{3}H]IAA metabolised at a higher rate in the mutant as compared to the wild type, suggesting that the mutation may be affecting IAA metabolism (Symons et al., 2002). However, application of IAA did not rescue the *bushy* phenotype which may not, therefore, be entirely attributable to the drop in IAA levels (Symons et al., 2002). The drop in IAA may not be governed solely by an upregulation of IAA metabolism, but also a downregulation of IAA biosynthesis. In light of the new techniques developed, it was possible to analyse putative IAA precursors in order to examine this possibility.

The lower IAA level makes *bushy* a key tool for the study of IAA biosynthesis: primarily, by examining the step IAAld to IAA – by aldehyde oxidase. Other studies have characterised three aldehyde oxidase genes present in pea (*PsAO1*, *2*, and *3*; Zdunek-Zastocka, 2008). Three protein isoforms have also been isolated in vegetative tissue, and more in seeds (Zdunek-Zastocka, 2010). One protein isomer is possibly involved with ABA production (*PsAOγ*), and another possibly associated with IAA, however, it is still unclear which protein dimer isoforms are encoded by which genes (*PsAOα*; Zdunek and Lips, 2001; Zdunek-Zastocka et al., 2004; Zdunek-Zastocka, 2008, 2010). Even if the mutation is not affecting IAA biosynthesis directly, the indirect effects make the mutant very interesting and invaluable in the discovery of key elements of IAA biosynthesis.

Figure 7.1. (L to R) 4-week-old plants of the isolines HL297M (homozygous mutant, *bsh*/*bsh*), HL297H (heterozygous mutant, *BSH*/*bsh*) and HL297W (WT, *BSH*/*BSH*). The mutant displays much shorter internodes, smaller and more numerous leaves, and a decreased stature. The mutation is dominant, but not completely so, as the heterozygote (centre) displays a phenotype intermediate between *bushy* and wild type.

Here the effects of the bushy mutation are further characterised. The endogenous levels of IAA precursors are studied, along with their abundance in different tissues within the mutant and wild type plants.

7.2. Materials and Methods

7.2.1. Plant Material and harvesting procedures

Seeds of the heterozygous HL297H (*BSH/bsh*) segregating in a 1:2:1 ratio of WT, heterozygote and *bushy* mutant plants were used for quantification experiments, grown as described (Chapter 2.1) for 6.5 weeks. Plant material was then harvested above the second-uppermost fully expanded leaf, and the entire apical portion used for quantitation. Because of the difference in weight of *bushy* plants, 3-4 apical portions were harvested for each replicate, instead of one as in the wild type. For each genotype, three replicates were harvested as described (Chapter 2.1.1). In other experiments, tissue of 4-week-old wild type and heterozygote plants were separated into internode and leaf material, from whole pea plants. Heterozygote plants were used on the basis that the main stem was a better comparison to the wild type, as opposed to *bushy* tissue, which was much smaller, with thinner and more branched internodes. All leaf and internode material was separated and used for hormone analysis. For all quantification studies, a known amount of appropriate internal standard was added to the extracts.

7.2.2. Preparation of extracts for GC/MS analysis

Once harvested and weighed, extracts were treated as described for tryptophan (Chapter 2.2.1), tryptamine (Chapter 2.2.2), IAAld, IEt (Chapter 2.2.5), and IAA (Chapter 2.2.6), and made ready for GC/MS analysis as described (Chapter 2.2.8).

7.2.3. GC/MS/MS analysis

Identification and quantification of the compounds were performed using gas chromatography combined with tandem mass spectrometry, as described in Chapter 2.3. For tryptamine, IAA IEt (including IAAld converted to IEt using sodium borohydride), and tryptophan, the GC/MS/MS conditions were as shown in Table 2.1. Compounds were all separable by GC/MS/MS, and eluted in the order tryptamine, followed 76 seconds later by IEt, (five seconds) IAA, and finally (4 minutes 29 seconds) tryptophan.
For all compounds measured, t-tests were performed comparing each of the three genotypes: *bushy*, heterozygote and wild type.

7.3. Results

7.3.1. Quantification of IAA precursors in bushy*, heterozygote and wild type pea plants.*

The putative IAA precursors tryptophan, tryptamine, IAAld and IEt were quantified in *bushy*, heterozygote and wild type pea shoot extracts. The identifications were based on GC/MS/MS analyses, using synthesised standards for reference.

IAA levels in *bushy* were significantly lower than those of the wild type, with the heterozygote an intermediate between them ($P<0.001$; Figure 7.2). The level of IEt in *bushy* was also noticeably, but not significantly, less than in the wild type (Figure 7.3). Conversely, the level of IAAld in the mutant was markedly increased as compared to the wild type: 52.8 (± 7.6) ng g FW^{-1}, compared with 22.9 (± 1.03) ng g FW^{-1} ($0.05>P>0.025$; Figure 7.4). Similarly to IAAld, tryptamine levels were also increased in the mutant ($P<0.001$; Figure 7.5). Tryptophan levels were not significantly different in the three genotypes ($P>0.09$; Figure 7.6), and the levels of IEt, and particularly tryptamine, were very low in all tissues.

7.3.2. Levels of precursors in leaves and internodes

In a separate experiment, the levels of IAAld and IAA were examined in the internodes and leaves of wild type and heterozygous *BSH/bsh* plants. Again, IAAld levels were significantly increased in the heterozygote ($0.05>P>0.01$; Table 7.1), more so in the internodes than the leaves (Figure 7.7). IAA levels in the internodes and leaves of the heterozygote were significantly different ($P<0.01$), but not as markedly as those of the wild type, which exhibited a huge build up of IAA in the internodes (198 ± 5.3 ng g FW^{-1}; Figure 7.8). IEt levels were reduced in the heterozygote, once again, but not significantly so in the leaves compared to the wild type ($P>0.1$), with more IEt present in the leaves than internodes of both heterozygote and wild type plants ($P<0.01$; Figure 7.9).

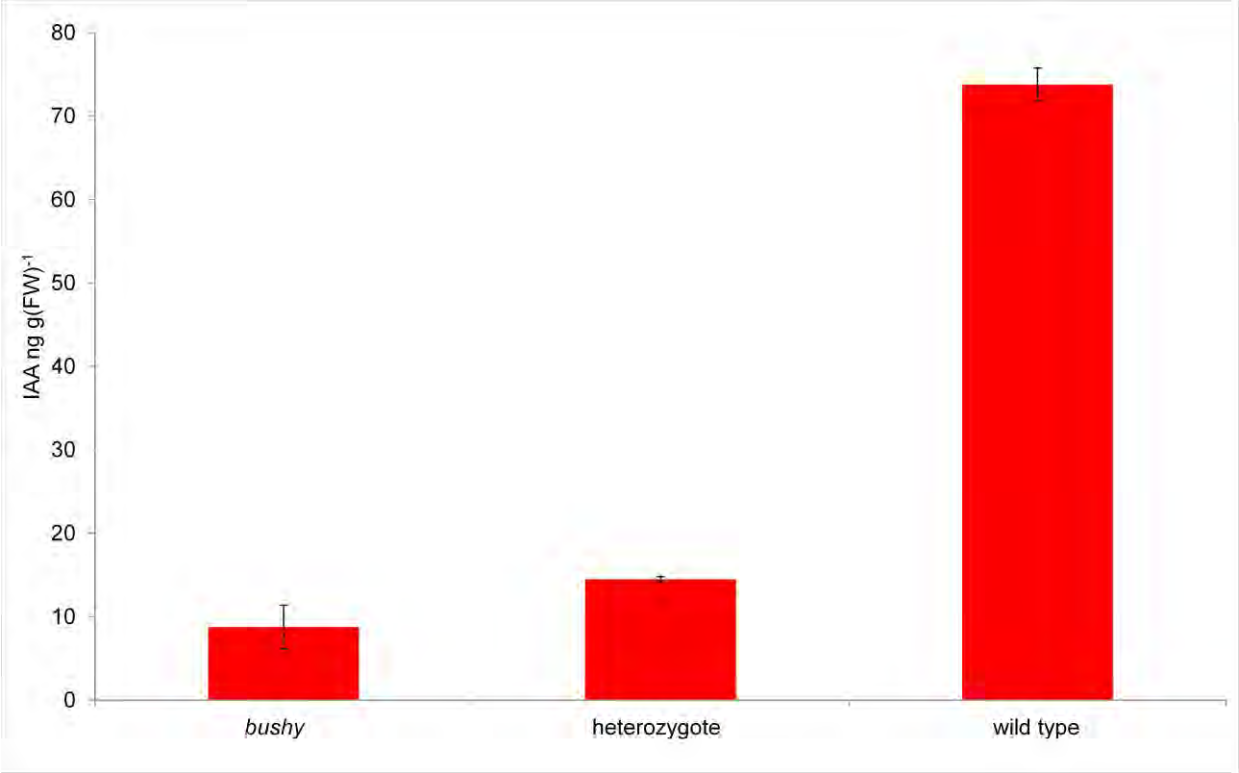

Figure 7.2. Levels of IAA (ng g [FW]$^{-1}$) in *bushy*, heterozygote and wild type, as analysed by GC/MS/MS. Shown are the mean levels of IAA ± standard error (n=3) for 6.5 week old plants.

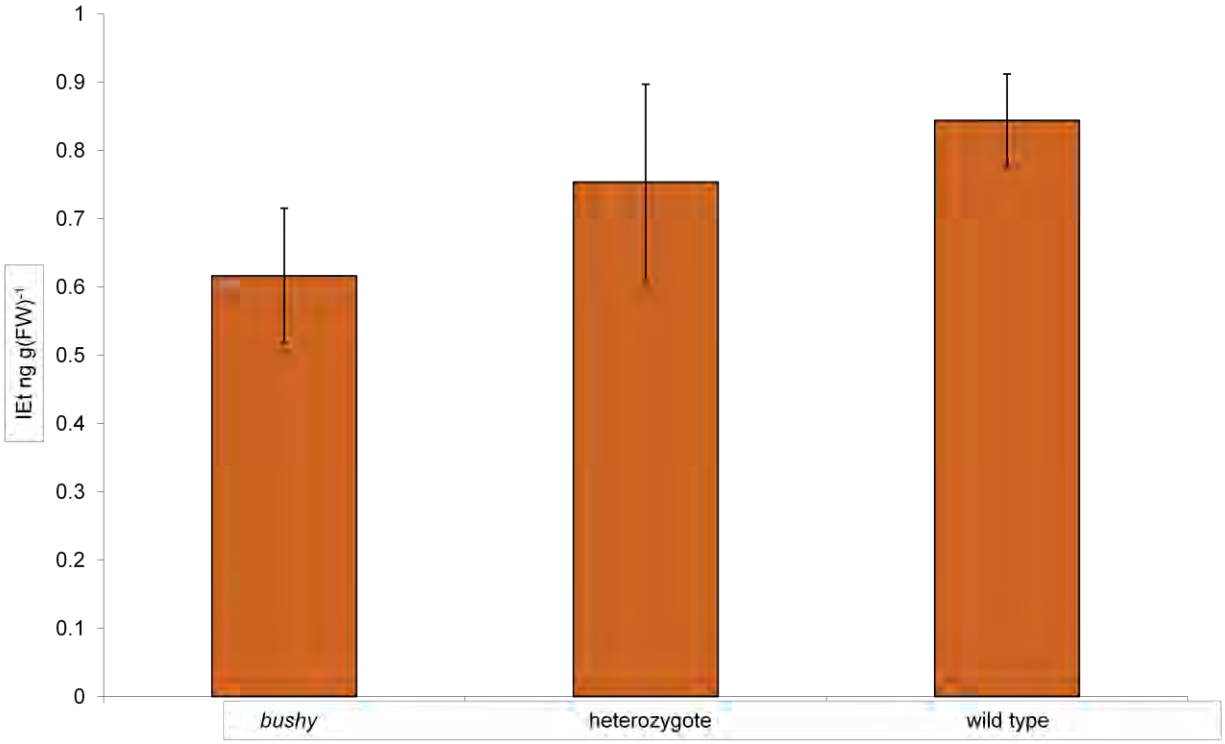

Figure 7.3. Levels of IEt (ng g [FW]$^{-1}$) in *bushy*, heterozygote and wild type, as analysed by GC/MS/MS. Shown are the mean levels of IEt ± standard error (n=3) for 6.5 week old plants.

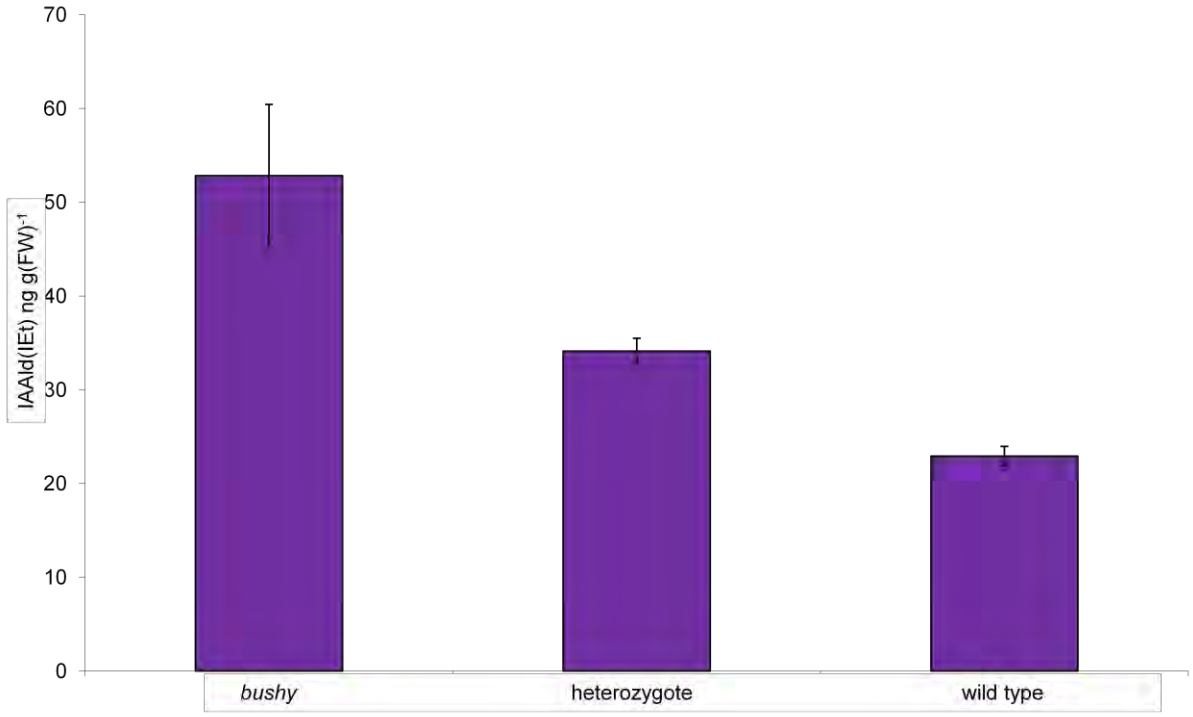

Figure 7.4. Levels of IAAld (converted to IEt; ng g $[FW]^{-1}$) in *bushy*, heterozygote and wild type, as analysed by GC/MS/MS. Shown are the mean levels of IAAld ± standard error (n=3) for 6.5 week old plants. This is the same experiment as in Figure 7.3 – where endogenous IEt was subtracted from total IEt after IAAld was converted.

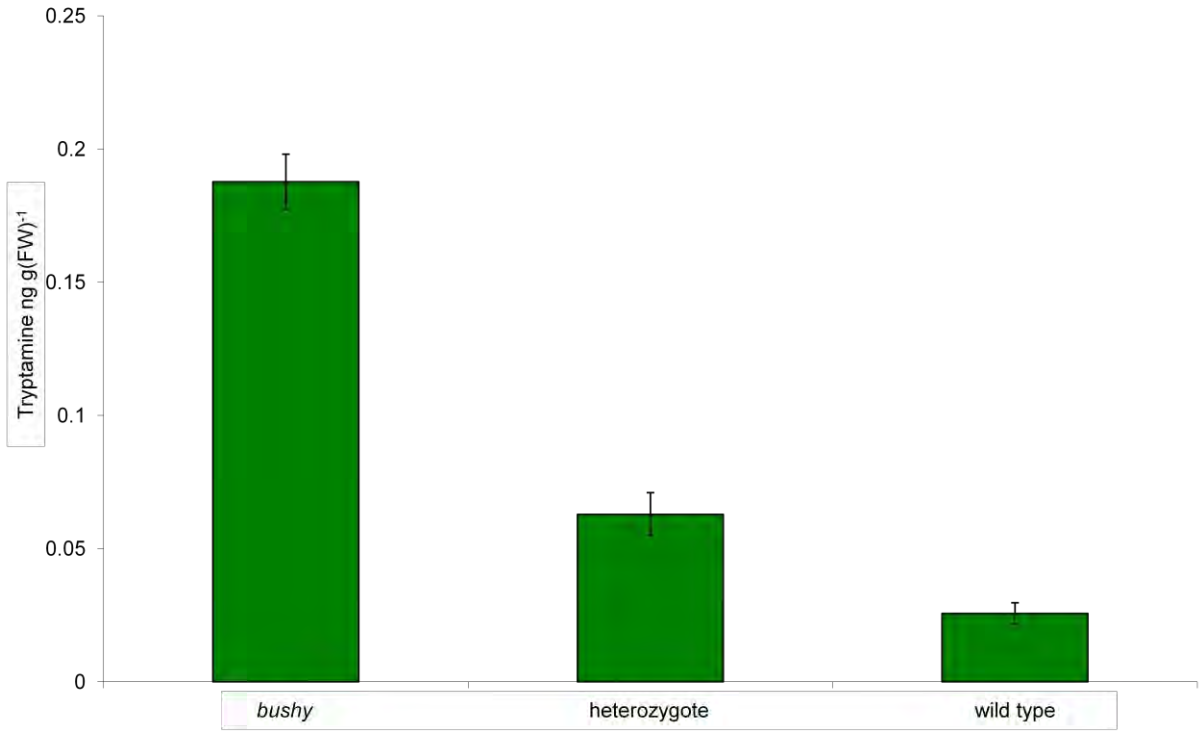

Figure 7.5. Levels of tryptamine (ng g [FW]$^{-1}$) in *bushy*, heterozygote and wild type, as analysed by GC/MS/MS. Shown are the mean levels of tryptamine ± standard error (n=3) for 6.5 week old plants.

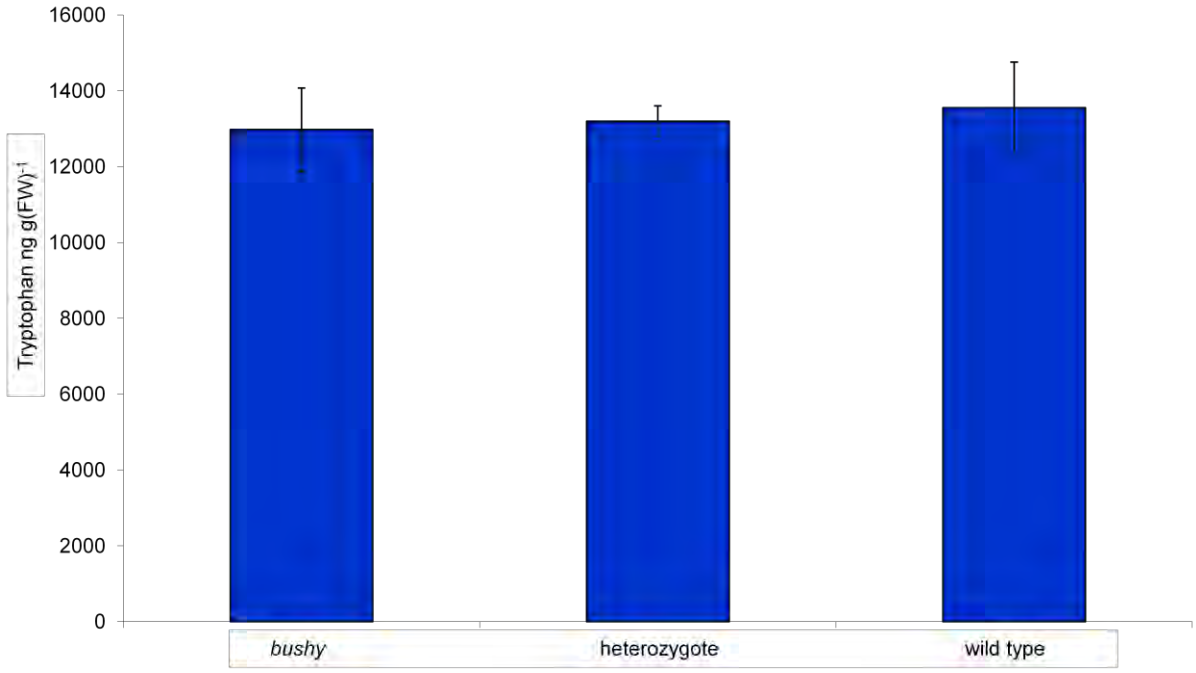

Figure 7.6. Levels of tryptophan (ng g [FW]$^{-1}$) in *bushy*, heterozygote and wild type, as analysed by GC/MS/MS. Shown are the mean levels of tryptophan ± standard error (n=3) for 6.5 week old plants.

Table 7.1. The results of t-tests, showing the significant differences between wild type and heterozygote *BSH/bsh* leaf and internode levels of IAAld, IAA and IEt. Data was obtained from 4-week-old plants depicted in Figure 7.7-7.9, where n=3.

t-test treatment	**IAAld**			**IAA**			**IEt**		
	t=	**P=**	**Significant**	**t=**	**P=**	**Significant**	**t=**	**P=**	**Significant**
WT leaf VS Internode	*0.88*	*>0.1*	*NO*	31.5	<0.001	YES	4.09	<0.05	YES
Heterozygote leaf VS Internode	*2.69*	*>0.05*	*NO*	4.6	<0.01	YES	3.7	<0.05	YES
WT VS Heterozygote leaf	3.72	<0.05	YES	5.16	<0.01	YES	*1.8*	*>0.1*	*NO*
WT VS heterozygote internode	15.07	<0.001	YES	34.9	<0.001	YES	5.85	<0.01	YES

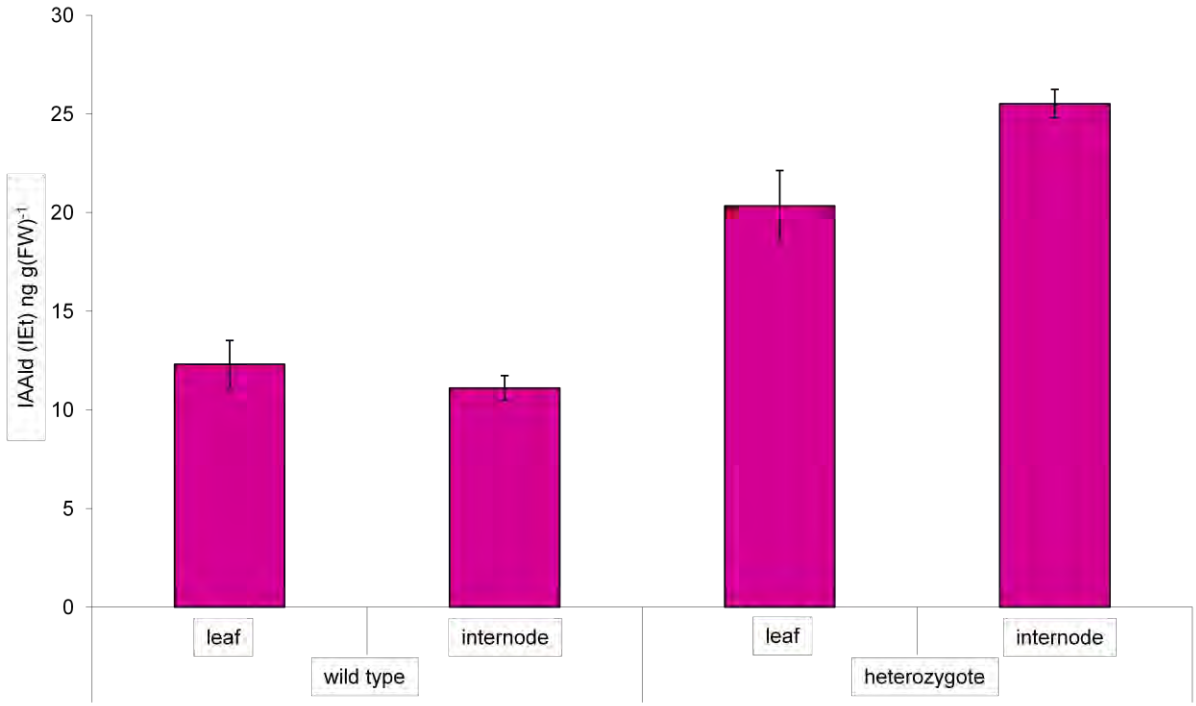

Figure 7.7. Endogenous levels of IAAld (converted to IEt; ng g $[FW]^{-1}$) in leaf and internode tissue of the wild type pea and heterozygous *BSH/bsh* lines. Shown are the mean levels of IAAld ± standard error (n=3) for 4 week old plants.

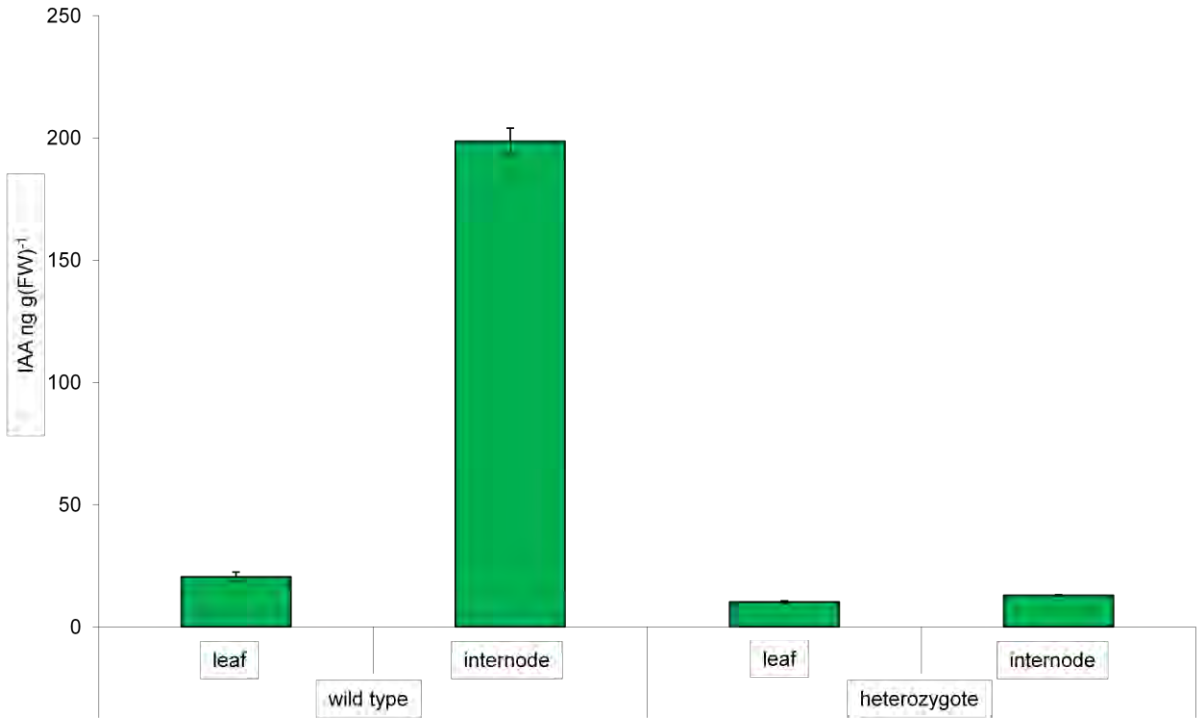

Figure 7.8. Endogenous levels of IAA (ng g [FW]$^{-1}$) in leaf and internode tissue of the wild type pea and heterozygous *BSH/bsh* lines. Shown are the mean levels of IAA ± standard error (n=3) for 4 week old plants.

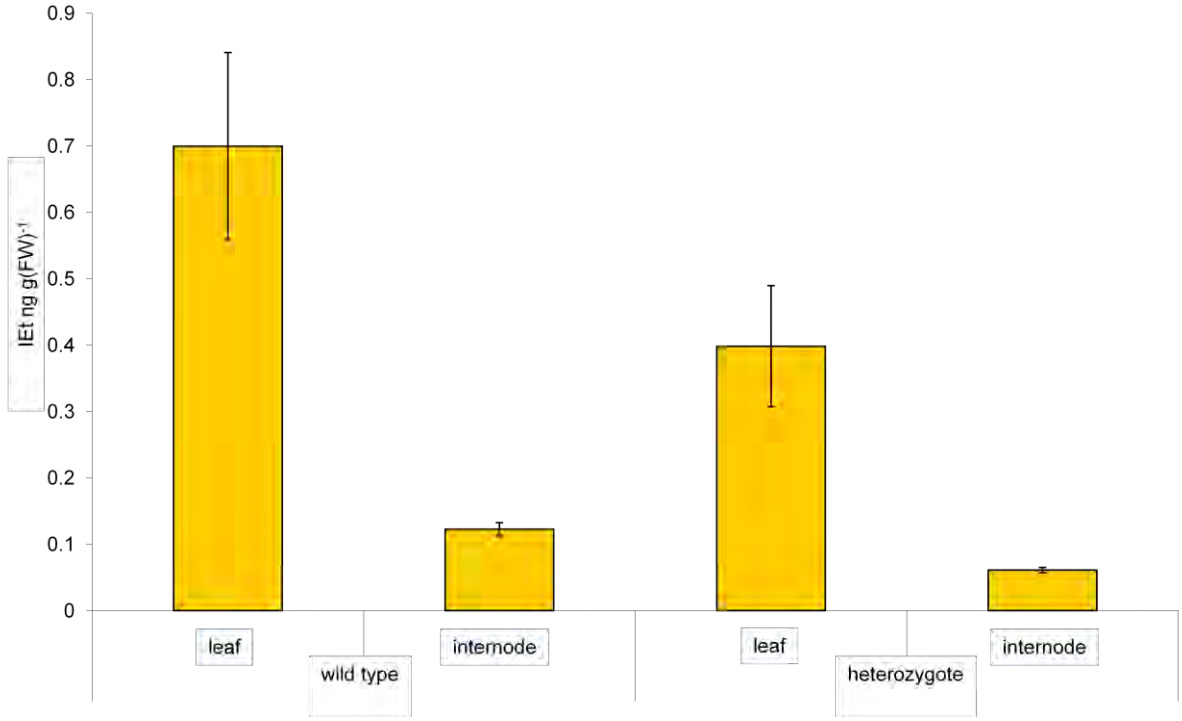

Figure 7.9. Endogenous levels of IEt (ng g $[FW]^{-1}$) in leaf and internode tissue of the wild type pea and heterozygous *BSH/bsh* lines. Shown are the mean levels of IEt ± standard error (n=3) for 4 week old plants.

7.4. Discussion

The results herein indicate that a tryptophan-dependent route to IAA operates in pea shoots. The increase in IAAld content in the presence of a decrease in IAA level, as in the *bushy* mutant, is strong evidence that within pea shoots, a biosynthetic route to IAA via IAAld is in operation. The question remains, via which tryptophan-derived intermediate does this pathway proceed? Evidence points to tryptamine as a possible pathway intermediate in roots (see Chapter 4; Quittenden et al., 2009), which is supported by the finding that tryptamine is also increased in *bushy* shoots. However, as yet, it is not possible to rule out the involvement of the IPyA pathway, also suggested to proceed via IAAld (Nonhebel et al., 1993; Bartel et al., 2001). As evidenced in Chapter 6, IAM appears to be absent from pea, or at least present at low levels, indicating that a pathway involving that compound is not active. Similarly, the compounds IAN and IAOx also appear not to be involved (Chapter 4).

The unaltered level of tryptophan in the *bushy* mutant suggests that whatever the mutation is affecting, it does so after this precursor. Another regulatory step, between IAAld and IEt via IAAld reductase (Rajagopal and Larsen, 1972), may also be affected in the mutant. IEt levels reflected IAA levels, with a reduction of the compounds shown in the mutant. It is possible that *bushy* may use stored IEt in order to produce more IAAld to further convert to IAA, even though this conversion is inhibited in the mutant. This would infer a regulatory system involving IAAld, IAA and IEt.

Interestingly, the drop in IAA and increase of IAAld was seen in both the leaves and internodes of the heterozygote as compared to the wild type, indicating that the findings do not represent a difference in only one tissue type, but the entire shoot. A dramatic drop of IAA was seen in the heterozygote internodes in comparison to the wild type. It has been shown that IAA is transported from the apical bud down the internodes through the basipetal transport stream, as well as longitudinally through cells (Morris et al., 2005). However, IAA may also be transported acropetally, after being synthesised in the leaves (Jager et al., 2007), or the roots (Ljung et al., 2001a; Quittenden et al., 2009). Perhaps in the heterozygote, and hence presumably the *bushy* mutant, the short supply of IAA means that wherever the hormone is produced, it is being turned over and not transported, as is suggested by the dramatic drop of IAA in the internodes of the heterozygote.

The *bushy* mutant will be a key tool in the further elucidation of the pathways to IAA biosynthesis, particularly in gaining insight into the step IAAld to IAA. Studies in our laboratories have found that mRNA expression of the previously characterised *PsAO1* (Zdunek-Zastocka, 2008) was lowered in *bushy* compared with the wild type (Sandra Davidson, unpublished data), suggesting that this aldehyde oxidase gene may be the one responsible for the conversion of IAAld to IAA in the IAA biosynthesis pathway of pea. This finding is in agreement with the original assessment of Zdunek-Zastocka et al. (2004), that *PsAO1* is involved in IAA synthesis due to its higher expression level in young leaves of pea. Interestingly, research in our laboratories has also indicated that although linked to, *PsAO1* is not allelic with *bushy* (Sandra Davidson, Robert Elliot, John Ross, unpublished data). It appears, therefore, that whatever the *bushy* mutation is affecting, either directly or indirectly, causes less conversion of IAAld to IAA in the IAA biosynthesis pathway, perhaps as a result of a lowered expression of the IAA-related aldehyde oxidase.

It has previously been shown that the application of IAA did not rescue the *bushy* phenotype (Symons et al., 2002). Therefore, the *bushy* phenotype may not be entirely attributable to the drop in IAA levels. It is possible that the mutation affects both the biosynthesis and metabolism of IAA, but may not be attributable to a direct mutation in either. Despite this, the altered levels of putative IAA biosynthesis intermediates provide evidence of their involvement in IAA synthesis, and allow further investigation into the mechanisms in place to both produce and regulate the hormone. Further experimentation must be carried out to determine the exact element (or elements) that the *bushy* gene is affecting, and the reasons for the resulting array of phenotypic and physiological symptoms.

CHAPTER 8

The Regulation of IAA Biosynthesis and the Effect of Wounding on IAA Content

8.1. Introduction

The amount of IAA in a plant is the result not only of its biosynthesis, but also of IAA degradation, conjugation, the hydrolysis of IAA conjugates (Sitbon et al., 2000), and transport into or out of specific areas (Normanly, 1997). It has also been suggested that conjugated IEt could be released, producing a pulse of IEt to contribute to the IAA pool via IAAld (Sandberg et al., 1987). Consequently, an ever-changing pool of IAA molecules could be present in any particular cell type or tissue. The balance between IAA biosynthesis, metabolism, and transport, known as IAA homeostasis, has been described as a dynamic process that responds to developmental and environmental signals (Michalczuk et al., 1992; Bandurski et al., 1995; Tam and Normanly, 1997, 1998; Ljung et al., 2001a). Hence, the pathways to IAA in plants are, most likely, under strict regulatory control to mediate such signals (Ribnicky et al., 1996). A complex network of regulation must occur in order to keep a constant pool of IAA available.

It has been previously suggested that developmental control of IAA synthesis involves the switching of IAA synthesis from a basal tryptophan-independent pathway to a tryptophan-dependent pathway in times of greater IAA requirements, such as in response to periods of high growth development or plant stress/wounding (Tam and Normanly, 1998; Bartel et al., 2001). The initial discovery of the tryptophan-independent pathway occurred with studies of the *orp* mutant in maize, which lacks the enzymes required to produce tryptophan (Wright et al., 1991). It is thought that the independent route branches off the shikimate pathway (the route to tryptophan) at indole-3-glycerol phosphate (IGP) or indole. However, there appears to be considerable species-to-species variation in the importance of this independent pathway, and Koshiba (1995) suggests that tryptophan auxotrophs (such as *orp*) are merely exhibiting normal minor pathways that become major when the biosynthesis of tryptophan is blocked. Furthermore, Muller and Weiler (2000) argue that in tryptophan auxotrophs, the high IAA content is in conjugated forms derived from IGP instability, not from the enzymatic

conversion of IGP to free IAA. Thus, the tryptophan-independent pathway may be an artifact of IGP instability, as opposed to a pathway to IAA synthesis in its own right.

No evidence of a tryptophan-independent pathway was found in maize endosperm and coleoptile tips in assay conditions (Koshiba and Matsuyama, 1993; Koshiba et al., 1995; Ilić et al., 1999; Glawischnig et al., 2000). However, maize seedling extracts do, apparently, produce IAA via an independent route (Östin et al., 1999). Similarly, *Arabidopsis* seedlings produce labelled IAA after feeding labelled anthranilate, but not tryptophan (Normanly et al., 1993), whereas *Arabidopsis* shoot and root explants successfully convert tryptophan to IAA (Müller *et al.*, 1998; Müller and Weiler, 2000).

Further confounding the dynamics of IAA biosynthesis and regulation, a growth phase-related shift of active IAA biosynthetic pathways may take place in many species. In *Pinus sylvestris* germinating seedlings, the synthesis of IAA from tryptophan occurs after day three, and the tryptophan-independent pathway begins after day six (Ljung et al., 2001b). Furthermore, in tomato fruit, a developmental phase change shift reportedly occurs from the tryptophan-dependent pathway in green fruit to tryptophan-independent synthesis in red fruit – although the IAA levels do not change (Epstein et al., 2002). Therefore, it appears as though the two pathways are a function of plant developmental stage and tissue, and that, depending on the stage of differentiation, cells may produce auxin through alternate pathways. However, there is a large gap in the research of tryptophan-dependent IAA biosynthesis in that no study has examined the regulation of IAA intermediates in response to tissue type, auxin application, or stress.

In addition to developmental regulation, it appears that there may be feedback regulation of IAA synthesis (Michalczuk et al., 1992). Research by Strader et al. (2011) suggests that a positive feedback system occurs in *Arabidopsis* through IBA. This is in agreement with the finding that in carrot cell cultures, the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) stimulates a several-fold increase in IAA content (Michalczuk et al., 1992). However, Ljung et al. (2001a) suggest a negative feedback system in *Arabidopsis*, where an initial increase of IAA level after treatment with naphthylphthalamic acid (an auxin transport inhibitor) induced feedback inhibition of IAA synthesis. Similarly, in carrot hypocotyls, exogenous application of IAA suppressed IAA biosynthesis, via the tryptophan-independent pathway, but did not

change tryptophan dependent synthesis (Ribnicky et al., 1996), although, once again, tryptophan-dependent pathway intermediates were not monitored.

Monitoring the effects of synthetic auxins, such as 1-naphthaleneacetic acid (NAA) and 2,4-D, can give valuable information about possible feedback regulation occurring in the plant. It appears as though the level of tryptophan is not a factor in determining the regulation of auxin production (Baldi et al., 1991), as the levels of the amino acid are so high in plants, and rarely change (as shown in Chapter 4), but it is possible that levels of intermediates in the tryptophan dependent pathway may have some impact on IAA regulation.

As mentioned previously, stressful conditions have also been shown to elicit a change in IAA biosynthesis in plants. This may be an ideal situation to monitor the regulation of IAA biosynthesis induced by a model biological treatment. In *Arabidopsis*, an increase in the tryptophan biosynthetic pathway enzymes is induced by lesion formation, pathogen induction, and abiotic elicitors (Zhao and Last, 1996), and a range of tryptophan-derived secondary products accumulate after infection with *Pseudomonas* (Zook, 1998; Hagemeier et al., 2001). However, if the levels of tryptophan do not elicit a change in IAA biosynthesis, as suggested by others (e.g. Baldi et al., 1991), these findings may not be relevant to the IAA biosynthesis pathway as the feedback steps would be after tryptophan.

Stress or wounding might affect IAA levels via intermediates after tryptophan in the biosynthetic pathways. Iino (1982) suggest that red light is a form of stress induction for *Zea mays*, and found a reduction in IAA levels in response to the treatment. Conversely, in bean, a transient action of tryptophan-dependent synthesis resulted in IAA accumulation after wounding (e.g. culture cells, tissue explants), but intact plants appear to use tryptophan-independent IAA biosynthesis (Sztein et al., 2002). Similarly, in *Lemna gibba*, stress in the form of altered temperature apparently changes the predominant pathway used to produce IAA (Rapparini et al., 2002). Furthermore, changes in aldehyde oxidase activity (the enzyme that converts IAAld to IAA) result from stress in pea (in the form of ammonium or saline treatment; Zdunek-Zastocka et al., 2004). It is potentially beneficial to plants that levels of tryptamine, a known neuroactive defense compound (Borowsky et al., 2001) might increase as a response to wounding. In poplar and tobacco plants, an increase in tryptophan decarboxylase (the enzyme that converts tryptophan to tryptamine) expression has been reported in response to herbivory, causing adverse effects in caterpillar and hornworm (Gill et

al., 2003). However, if the IAA pulse exhibited in some plants after wounding, such as *Arabidopsis* leaves (Ljung et al., 2001a) is due to an increase of tryptamine levels, this may cast doubt on the proposed rate-limiting function of Arabidopsis *YUCCA* (the enzyme that converts tryptamine to *N*-hydroxytryptamine in the *Arabidopsis* tryptamine pathway to IAA; Zhao et al., 2001). Furthermore, the tryptophan-dependent pathway intermediates themselves have not been monitored in any of these studies, casting doubt on the substance of some of these claims.

Because many of the studies conducted on the tryptophan-dependent IAA biosynthetic pathway have been carried out using plant parts as opposed to whole plants, this wounding phenomenon could cause a major difficulty in ascertaining whether tryptophan-dependent pathways are actually operative in whole plants (Ljung et al., 2001a). That is the reason for using whole plants as opposed to *in vitro* or plant part analysis throughout this research.

In this study, pea plants were incubated with exogenous IAA, the synthetic auxin NAA, and the auxin signaling inhibitor 2-(p-chloro-phenoxy)-isobutyric acid (PCIB; Oono et al., 2003), in order to determine whether regulatory control of the tryptophan-dependent auxin biosynthetic pathway was exhibited in response to these stimuli. Furthermore, the effect of wounding on the levels of IAA and intermediates in the pathway were examined.

8.2. Materials and Methods

8.2.1. The effects of exogenous auxin or auxin signaling inhibitor application

Pea plants of the line 107 were grown in Murashige and Skoog (1962) medium as described in Chapter 2.1.2, for one week. Subsequently, the seedlings were transferred into aerated water, containing 1, 10 or 100 μM NAA, or in separate experiments: 10 μM IAA, and 50 μM PCIB, or solely water, and left for 24 hours. The root material was then harvested, homogenized and filtered as described (Chapter 2.2), and analysed for IAA, IAAld, and IEt (Chapter 2.2, 2.3).

8.2.2. The effect of wounding on IAA levels

Plants were grown as described for quantitation studies in Chapter 2.1. After three weeks of growth, the plants were subjected to wounding, by slicing with a razor blade twice on the stipules of the apical portion, and also twice on the uppermost leaflets. After one hour, the wounded portions were harvested, and homogenized and filtered as described (Chapter 2.2).

The subsequent extract was analysed for tryptophan, IAA, IAAld and IEt as described (Chapter 2.2, 2.3).

In another study, pea plants grown in the same conditions for three weeks, as described above, were wounded (as previously outlined), or left whole, and harvested at intervals of one, three, six, nine, and 12 hours to determine whether the wounding response was time-dependent. Extracts from this study were analysed for IAA and IEt as described in Chapters 2.2 and 2.3.

8.3. Results

8.3.1. The effects of exogenous IAA application on levels of IAA and precursors to IAA

After incubating pea roots with IAA, a large build up of IAA was seen endogenously. This was to be expected, as the analysis did not permit the separation of synthetic and endogenous IAA – the increased IAA levels were a result of the uptake of the IAA in which the plants were incubated. However, IAAld levels were dramatically higher in the IAA-fed plants, and IEt levels were approximately 50% lower in the treated plants (Figure 8.1). There was no replication within this experiment.

8.3.2. The effects of NAA treatments on IAA and precursors to IAA

After incubating pea roots with three different levels of NAA, a clear pattern emerged in the levels of IAA and IAAld (Figure 8.2 and Figure 8.3). With increasing NAA, IAA levels decreased significantly ($P<0.05$). With 1 μM NAA, IAAld levels increased noticeably compared to the control, and increased significantly with 10 μM NAA ($P<0.05$); however with 100 μM NAA, IAAld levels appeared to drop back to control levels. IEt levels were reduced slightly compared with control levels, with increasing NAA fed ($P<0.05$ between control and 100 μM NAA; Figure 8.4).

8.3.3. The effects of PCIB treatments on IAA levels

After incubation with PCIB, pea seedlings exhibited a drop of almost 50% in IAA levels ($P<0.001$; Figure 8.5).

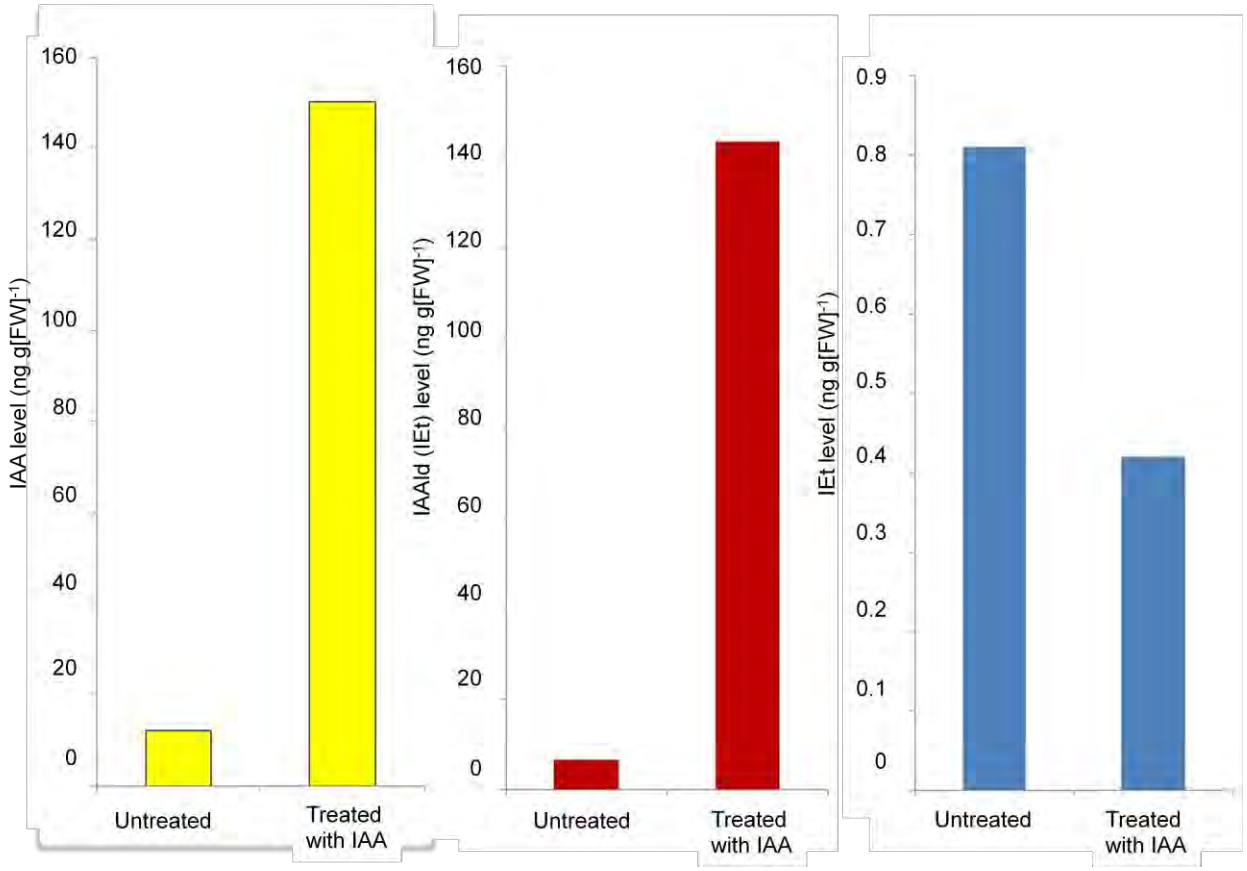

Figure 8.1. Levels in ng g(FW)$^{-1}$ of IAA (yellow), IAAld (converted to IEt, red) and IEt (blue) in pea roots incubated with or without 10 µM IAA for 24 hours, as analysed by GC/MS/MS (n=1). The level of IAAld was calculated by subtracting endogenous IEt from total IEt after IAAld was converted.

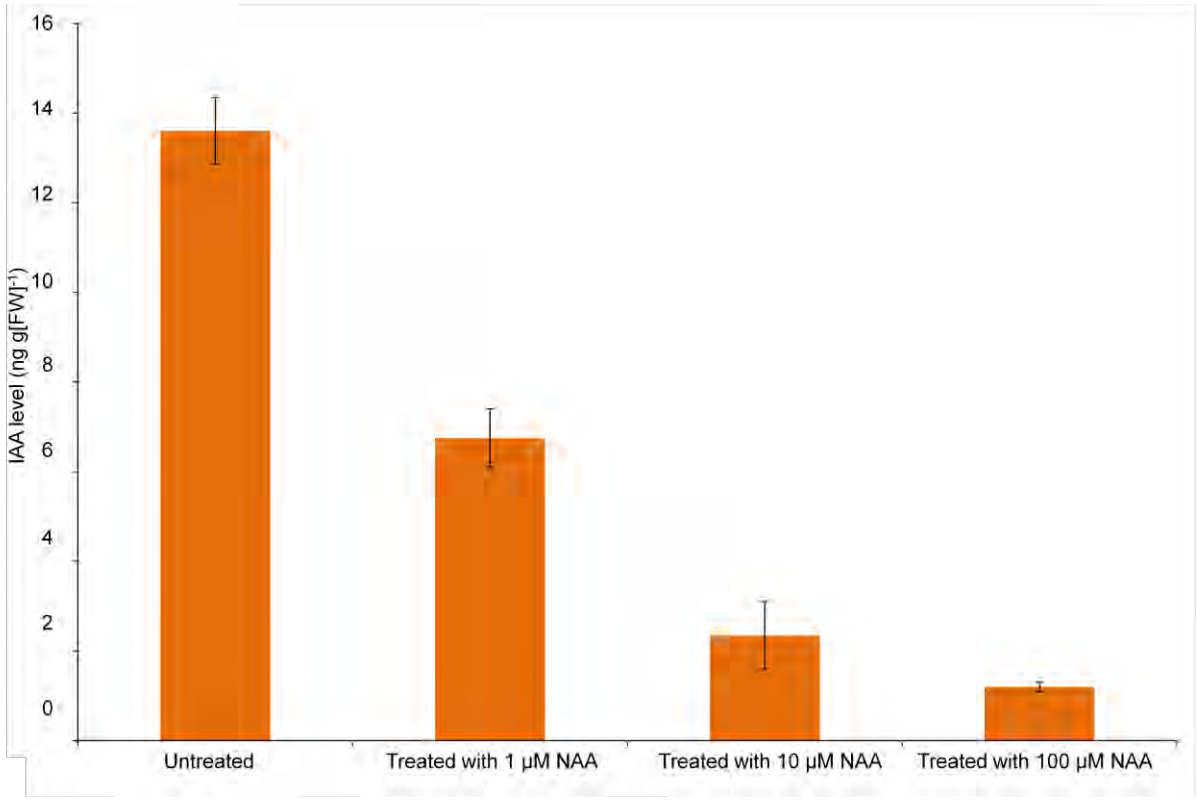

Figure 8.2. The levels of IAA (ng g[FW]$^{-1}$) in pea roots after incubation with 0, 1, 10 and 100 µM of NAA for 24 hours, as analysed by GC/MS/MS. Shown are the means ± standard errors (n=2).

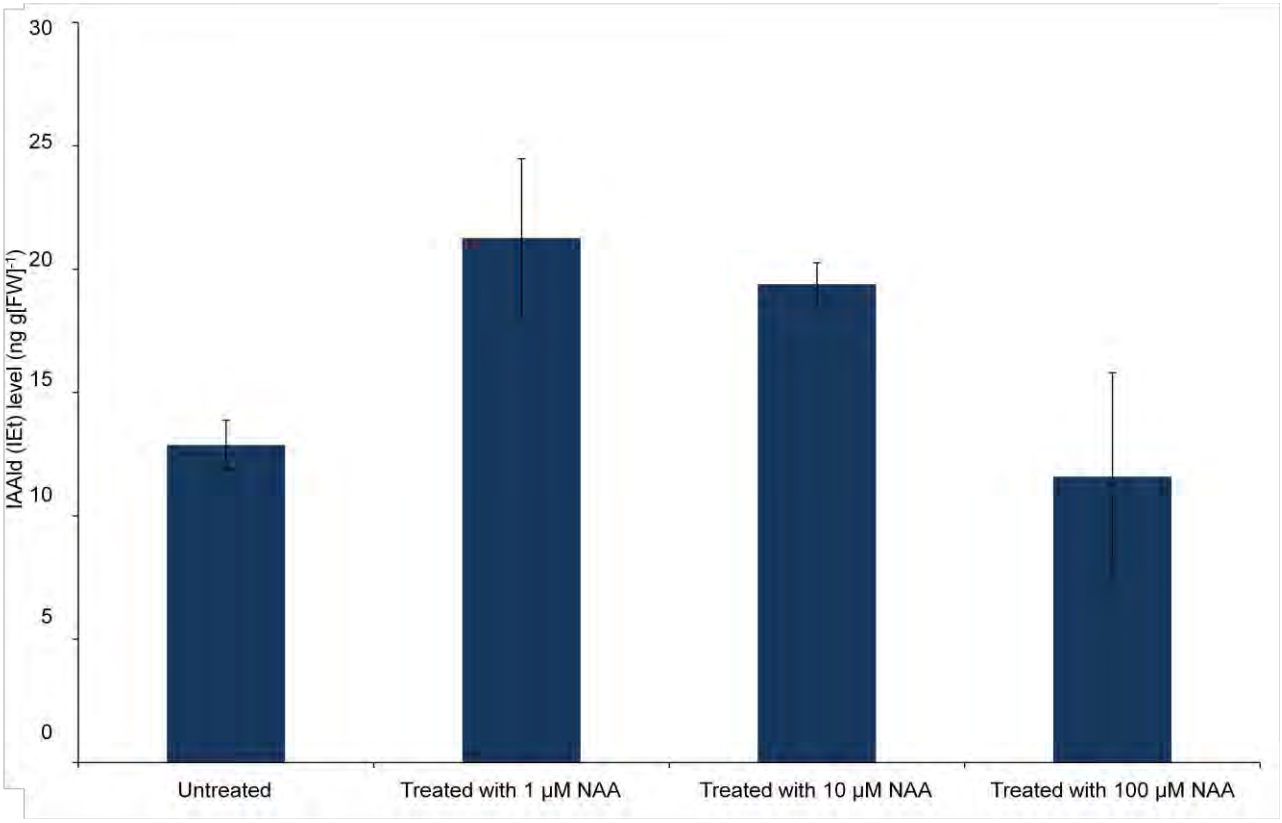

Figure 8.3. The levels of IAAld (converted to IEt; ng g[FW]$^{-1}$) in pea roots after the incubation with 0, 1, 10 and 100 µM of NAA for 24 hours, as analysed by GC/MS/MS. Shown are the means ± standard errors (n=2). The level of IAAld was calculated by subtracting endogenous IEt from total IEt after IAAld was converted.

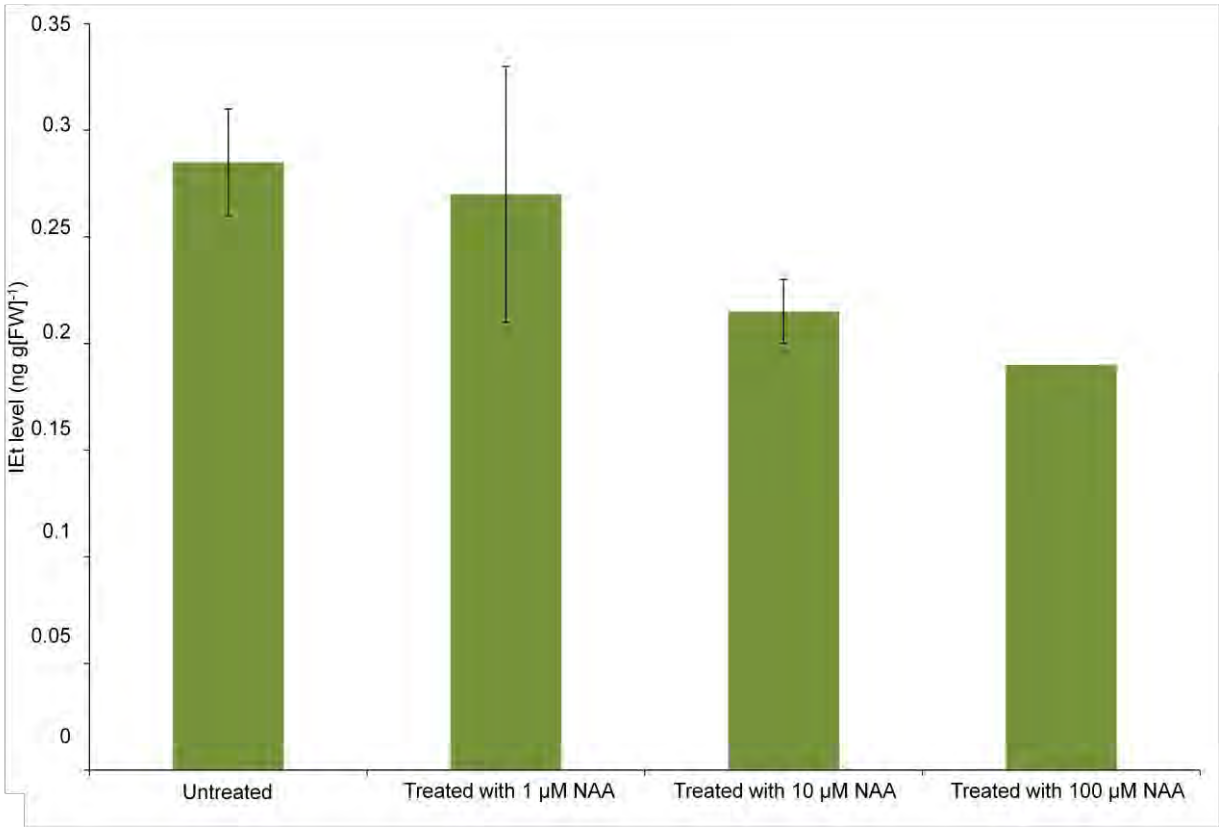

Figure 8.4. The levels of IEt (ng g[FW]$^{-1}$) in pea roots after the incubation with 0, 1, 10 and 100 µM of NAA for 24 hours, as analysed by GC/MS/MS. Shown are the means ± standard errors (n=2).

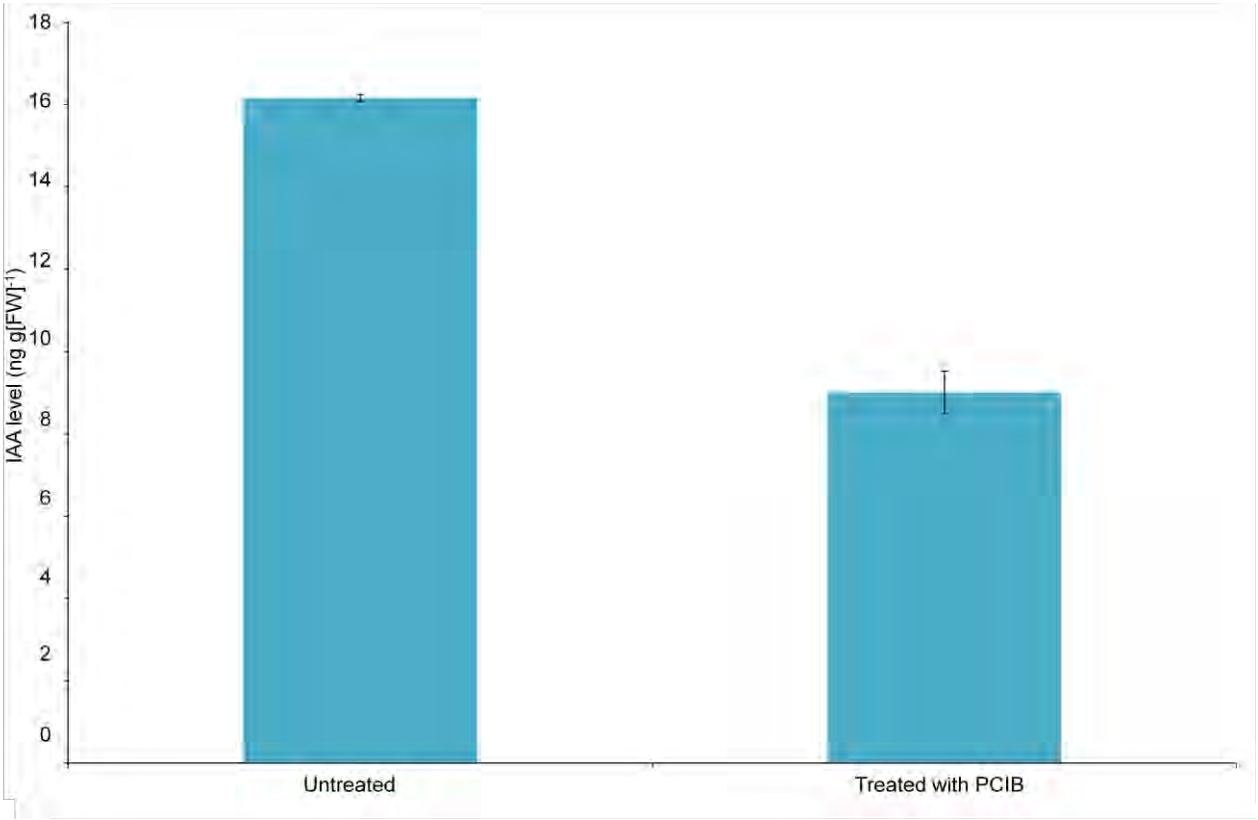

Figure 8.5. The levels of IAA (ng g[FW]$^{-1}$) in pea roots after the incubation with 50 µM PCIB for 24 hours, and those left untreated, as analysed by GC/MS/MS. Shown are the means ± standard errors (n=2).

8.3.4. The effect of wounding on levels of IAA, and precursors to IAA

An initial study found that wounding elicited no response in levels of IAA or of the putative precursors tryptophan, IAAld and IEt, in pea seedlings, after one hour (Table 8.1).

In order to investigate possible longer-term effects of wounding, in another experiment, IAA and IEt were measured in wounded and unwounded plants over a series of time points (1, 3, 6, 9, and 12 hours). As shown in Figure 8.6, a slight drop in IAA was found in wounded plants after six hours ($P<0.02$), remaining low in comparison to the control until the 12 hour time point ($P<0.02$). In contrast, the level of IEt in wounded plants was far higher than that of the control after three hours ($P<0.02$), and continued to accumulate until nine hours ($P<0.05$), after which the levels appeared to drop, but remained significantly different to the control ($P<0.01$; Figure 8.7).

Table 8.1. The levels (ng g $[FW]^{-1}$) of tryptophan, IAA, IAAld and IEt in wounded or unwounded pea plants after one hour (n=1) as analysed by GC/MS/MS. Data is representative of one experiment. The level of IAAld was calculated by subtracting endogenous IEt from total IEt after IAAld was converted.

Compound	**Unwounded (ng g$[FW]^{-1}$)**	**1 hour after wounding (ng g$[FW]^{-1}$)**
Tryptophan	241209	252120
IAA	37.5	30.8
IAAld (converted to IEt)	9.06	9.56
IEt	0.3	0.34

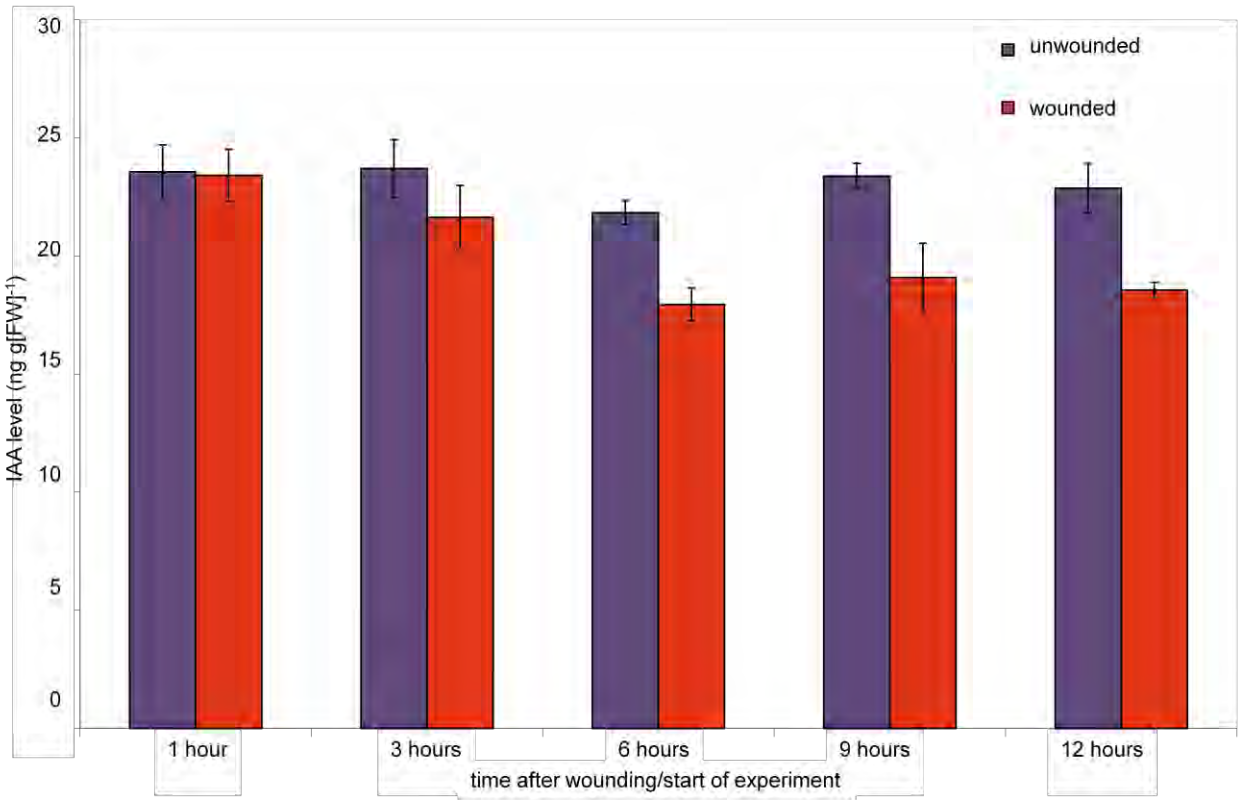

Figure 8.6. The levels of IAA (ng g[FW]$^{-1}$) in pea shoots with or without wounding as analysed by GC/MS/MS. Pea plants were harvested to determine IAA levels one, three, six, nine and twelve hours after wounding/experiment initiation. Shown are the means ± standard errors (n=3). Data represented here is from the same experiment as shown in Figure 8.6.

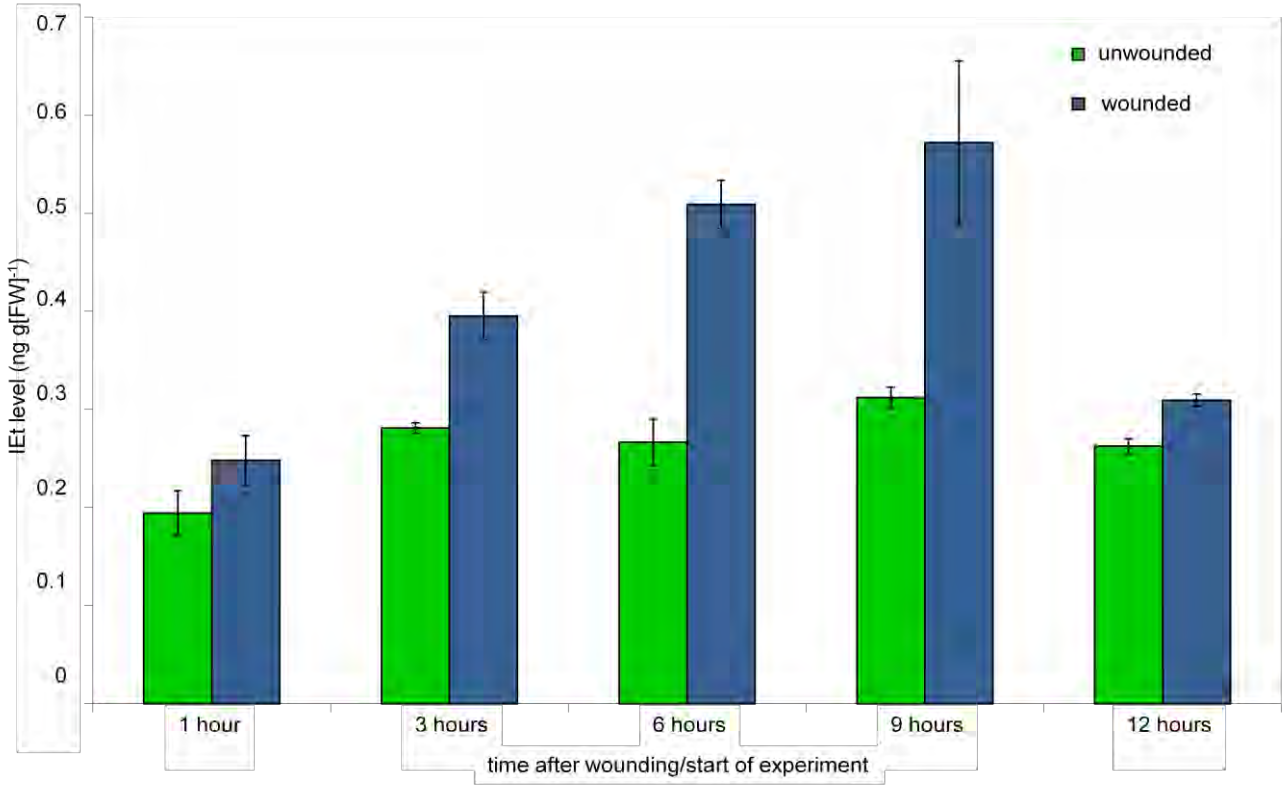

Figure 8.7. The levels of IEt (ng g[FW]$^{-1}$) in pea shoots with or without wounding as analysed by GC/MS/MS. Pea plants were harvested to determine IEt levels one, three, six, nine and twelve hours after wounding/experiment initiation. Shown are the means ± standard errors (n=3). Data represented here is from the same experiment as shown in Figure 8.5.

8.4. Discussion

8.4.1. The regulation of IAA biosynthesis

The results herein indicate that treatment with either IAA or NAA increases the levels of IAAld in pea roots. Furthermore, after NAA incubation, IAA levels decreased significantly. Therefore, synthetic auxin application appears to reduce the production of IAA by slowing the conversion of IAAld to IAA, resulting in the build-up of the intermediate. These results suggest a negative feedback mechanism for the regulation of tryptophan-dependent IAA biosynthesis in roots, governed by the levels or requirements of the compound itself, in agreement with the findings of Ribnicky et al. (1996) and Ljung et al. (2001a), in carrot and *Arabidopsis*, respectively. If IAA biosynthesis was controlled solely by a tryptophan-independent pathway, presumably no build up of IAAld would be expected – therefore, it appears as though tryptophan-dependent synthesis of IAA is operative in pea. This is the first time the intermediates to IAA have been monitored in response to synthetic auxins in pea.

It has been found previously that treatment of pea plants with 2,4-D inhibits IAAld oxidase, the enzyme responsible for this step (Kutáček and Terziivanova-dimova, 1991), and up regulates IAAld reductase, the enzyme responsible for the conversion of IAAld to IEt, in cucumber (Bower et al., 1978). However, no discernable increase of IEt was found in either IAA or NAA feeding studies; in fact, a decrease in the level of this compound was seen, relative to the controls. It is possible that not enough time was given for the IAAld to be converted to IEt for storage. Interestingly, as described in Chapter 7, the low-IAA pea mutant *bushy* also exhibits high IAAld levels, with reduced IEt levels.

The compound PCIB is thought to impair the auxin signaling pathway by regulating Aux/IAA protein stability. In *Arabidopsis*, PCIB antagonizes exogenous applied auxin by inhibiting the upstream auxin-signaling events, suggesting that PCIB reduces the cellular response to auxin (Oono et al., 2003; Biswas et al., 2007). However, we cannot be confident that the action of PCIB is restricted entirely to IAA response, but may be affecting other IAA-related functions. Biswas et al. (2007) suggest that the reduction of cellular response to internal auxin by PCIB could abolish feedback regulation, and lead to an over compensation with an ectopic cellular auxin accumulation that ultimately lowers root growth. Therefore, based on this assumption, it would be expected that plants incubated with PCIB would produce more IAA to compensate for the lack of response within their tissues, as has been shown previously in *Arabidopsis* (Biswas et al., 2007). Here, however, PCIB lowered the

level of IAA in pea root tissue. Therefore, the results of the PCIB incubation experiment do not fit with the model of IAA regulation by negative feedback. As the specificity of PCIB to IAA signaling and response has not been exhaustively tested, PCIB may not have been suitable for this style of IAA biosynthesis feedback study. In future, an IAA biosynthesis inhibitor could be sought, and used to further determine the homeostatic control of IAA.

8.4.2. The effect of wounding on IAA biosynthesis and regulation

The effect of wounding on IAA levels, and therefore the hormone's regulation, was also studied. After six hours a significant drop in IAA was found in wounded pea shoots, and IAA levels remained low until the termination of the experiment, at twelve hours. Conversely, IEt levels were significantly higher in the wounded plants between three and nine hours, before dropping back to control-like levels at twelve hours. In response to wounding, it appears that IAA synthesis is downregulated, and IEt synthesis is upregulated.

Wounding has been found previously to induce a transient action of the tryptophan-dependent pathway to produce higher IAA levels for cell proliferation and regeneration in *Arabidopsis* and bean (Müller and Weiler, 2000; Sztein *et al.*, 2002). The current research indicates that IAA levels decrease after wounding, in direct contradiction to the Sztein (2002) result. The method for wounding used in the current study was a standard wounding technique (Sun et al., 2006).

Previous research has suggested that there may be tissue-dependent changes in IAA regulation. In *Arabidopsis*, the process of dissecting plant parts in order to perform deuterium-incubation studies uncovered that dissected young leaves produced more IAA than intact leaves. Conversely, excision of roots lowered the amount of IAA synthesized in comparison to intact plants (Ljung et al., 2001a). The current findings are in agreement with the latter: that wounding results in a decrease in IAA production, even though shoots, not roots, were analysed in the present study.

It is possible that pea plants of this line do not possess the stress-related defensive compounds that other plants, such as *Arabidopsis* (Müller and Weiler, 2000) and bean (Sztein et al., 2002) appear to have in order to increase IAA levels upon wounding (however, the line used is wild type in most respects). It is feasible that the wound-induced changes in IAA levels found in other species (such as *Arabidopsis* leaf tissue; Ljung et al., 2001a) are due to an

increase in the indole glucosinolates, compounds known to be involved in defense against herbivory, that are found only in the Brassicas (Sugawara et al., 2009). This would result in an influx of auxin through IAN (a metabolite of the indole glucosinolates) via nitrilase, another known wound-activated enzyme (Quirino et al., 1999), as a secondary effect of wounding. Further study on the wounding response is needed to clarify these findings.

Pea shoot tissue exhibited a reduction in IAA level, whereas IEt was found to increase in response to wounding. This is in contradiction to the wounding response in bean, where IAA levels are increased (Sztein et al., 2002). Presumably, the process of IAAld being reduced to IEt is upregulated by wounding. It is possible that this decrease in IAA is in an effort by the plant to reduce growth in the affected part, and possibly to induce branching, in order to create more plant material in non-wounded areas to compensate for the loss of the wounded tissue (resulting from a decrease in IAA). In order to test this hypothesis, the known branching-related hormone, strigolactone (Ferguson and Beveridge, 2009) could be measured in both wounded and whole tissue.

Both uncovering that wounding pea shoot tissue induces a decrease in IAA, and that application of IAA or NAA elicit a reduction in IAA level, sheds light on the fact that IAA biosynthesis is a regulated process in pea. Further study must be undertaken to explore the molecular mechanisms behind this regulation, and its implications in terms of IAA biosynthesis and homeostasis.

CHAPTER 9

Conclusions

The processes by which auxin (IAA) is produced *in vivo* have yet to be fully elucidated, and are a topic of debate amongst plant biologists (see Chapter 1). Currently, the specific pathway to IAA has not been completely characterised in any one plant species, and it is possible that a combination of pathways exists. In addition, within a particular species, different tissues and developmental stages appear to affect which pathway to auxin predominates. Compounding this complexity, many of the pathway steps that have been defined have genetic redundancy, with more than one gene encoding the relevant enzyme.

In this thesis, auxin synthesis in pea has been examined, specifically in young roots and three-week-old shoots. A physico-chemical approach has been used to detect and monitor auxin and its potential precursors in wild type and a low-IAA mutant of pea, *bushy*. In addition, the metabolic fates of IAA precursors have been examined, as well as possible feedback mechanisms. A new pathway to IAA by tryptamine has been proposed.

New techniques were developed for the isolation and quantitation of potential precursors of IAA by GC/MS. Some of the compounds are labile and degrade quickly, which makes them difficult to analyse; however, techniques were developed for the isolation and detection of IAAld, IEt, tryptamine, tryptophan, IPyA, IAN and IAOx by GC/MS, and IAM by LC/MS. Within pea tissue, IAAld, IEt, tryptophan, and tryptamine were isolated and quantified, and the absence of IAM, IAN and IAOx was demonstrated. A technique was also developed for the uptake of precursors into pea roots in aseptic conditions.

Using these techniques, it was demonstrated that key elements of the tryptamine pathway can be operative in pea roots. The tryptamine pathway in peas differs from tryptamine pathways reported in other species in that tryptamine appears to be directly converted to IAAld as opposed to *N*-hydroxytryptamine or IAOx, as suggested by others (Figure 9.1; Zhao et al., 2001; Lau et al., 2008). No evidence of endogenous IAOx or IAN was found in peas, and labelled tryptophan was not metabolised to either IAOx or IAN in pea roots, indicating that the involvement of these compounds in IAA synthesis in vegetative pea tissue can be

tentatively dismissed, consistent with the conclusions of Sugawara et al. (2009). The new pathway to IAA by tryptamine in peas is represented in Figure 9.1.

Although IPyA was not isolated from pea tissue, the lability of this compound means it is possible that there is an endogenous pool, with the techniques being inadequate to determine its presence. However, the finding that tryptophan is converted to IAAld implicates either or both the tryptamine and IPyA pathways, as IAAld is a common intermediate: it was not possible to separate the two in this aspect. Further study must be undertaken on the metabolic fate of IPyA *in vivo*, and its involvement in IAA biosynthesis in vegetative pea tissue.

The IAM pathway to IAA, although well established in bacteria and recently observed in some higher plants (Lehmann et al., 2010), appears not to operate in pea roots. No accumulation of labelled IAA was observed after incubation of pea roots with deuterium-labelled IAM, and furthermore, labelled tryptophan was not converted to IAM. Some evidence points to the IAM pathway playing a role in IAA synthesis in *Arabidopsis* (Pollmann et al., 2009); however, it appears as though the pathways to IAA in this species are markedly different to those in pea. Not only was no endogenous IAM found in pea, but there was also no evidence for IAOx and IAN, two intermediates postulated to be involved in a pathway before IAM in *Arabidopsis* (Sugawara et al., 2009).

In this thesis, an examination of the low IAA pea mutant, *bushy*, was undertaken. It is apparent that regardless of the primary lesion in *bushy*, the mutation either directly or indirectly alters the biosynthesis of IAA. In shoots, both tryptamine and IAAld levels were significantly higher in the mutant than the wild type (as depicted in Figure 9.2). This finding gives further strength to the conclusion that the tryptamine pathway to IAA is operative in peas. However, the finding that IAAld levels are higher in the mutant, on its own, does not differentiate between the tryptamine and IPyA pathways, both of which may operate via IAAld. As the levels of tryptophan are similar in the mutant and the wild type, it appears as though any regulation of IAA synthesis occurs after that compound has been metabolised to other intermediates.

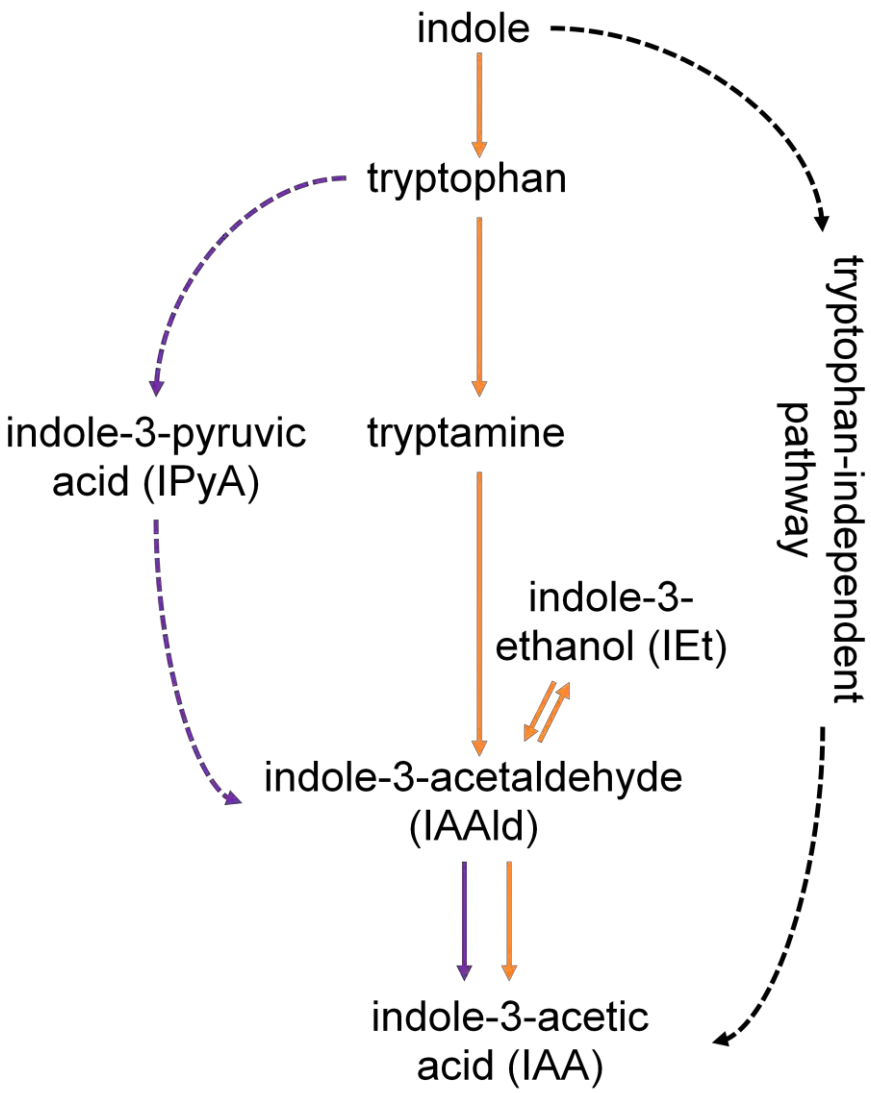

Figure 9.1. The proposed pathways to IAA biosynthesis in pea. Shown are the tryptamine (orange), and IPyA (purple) pathways. The tryptophan-independent pathway (black) may also operate. The dashes represent steps that have not been studied here, but which could occur in peas.

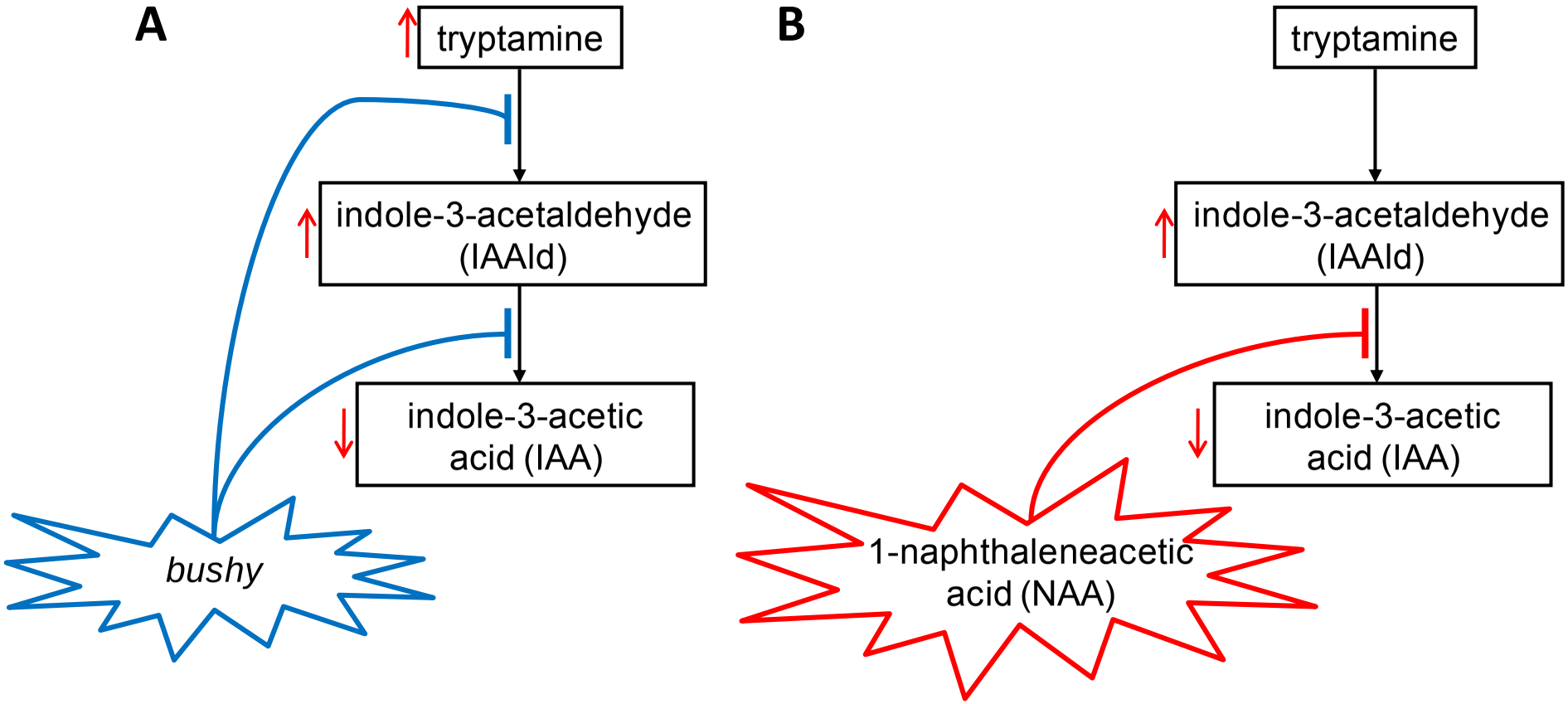

Figure 9.2. The proposed negative feedback regulation of IAA biosynthesis in vegetative pea tissue. **A** shows the sites of regulation that the *bushy* mutant is acting on (blue), with IAA levels lower, and IAAld and tryptamine higher in the mutant than wild type. **B** shows the site where NAA acts, causing lower IAA levels, and higher IAAld levels – by slowing the conversion of IAAld to IAA.

In terms of the regulation of IAA, it is proposed here that a negative feedback system is operative in pea roots; the production of IAA is reduced by slowing the conversion of intermediates to the hormone when auxin levels are high (such as when auxin is applied). This finding is in agreement with Ribnicky et al. (1996) and Ljung et al. (2001a), in carrot and *Arabidopsis*, respectively. However, those studies did not involve analyzing the levels of precursors to IAA. The findings herein were novel in that they implicate IAAld in the regulation of IAA biosynthesis, with the levels of the compound higher in roots incubated with either IAA or NAA. It seems that the most significant step affected by external auxin application is that from IAAld to IAA (Figure 9.2).

Wounding resulted in a decrease in IAA level, and an increase in IEt in pea shoots. The transport of IAA from the apical bud to high-growth regions is a well studied process (Davies, 2004). The decrease in IAA at the wound site may be an attempt to reduce growth in the affected part, and possibly to induce branching, in order to create more plant material in non-wounded areas to compensate for the loss of the wounded tissue. Interestingly, these results are in contradiction to the result of Stein et al. (2002), where bean (a close relative of pea) demonstrates an increase in IAA level in response to wounding. It is likely that this wounding phenomenon is under strict regulatory control, and brings to light the importance of using intact plants in determining the active pathways to IAA biosynthesis *in vivo*.

It appears that, in pea plants, the involvement of specific IAA biosynthesis pathways is dependent on tissue type. Studies on pea seeds have indicated that the tryptamine pathway is not operative (Tivendale et al., 2010), although, evidence has been presented here that both roots and shoots use the tryptamine pathway to IAA synthesis. The pattern in the levels of IAA precursors in roots after synthetic auxin incubation is comparable to the pattern of the same intermediates in shoots of the low IAA mutant, *bushy*. IAA levels are decreased in pea roots after NAA incubation, and in shoots of the low-IAA mutant, *bushy*. The levels of IAAld are increased in *bushy* shoots, and also in pea roots after NAA incubation (represented in Figure 9.2). Similarly, the level of IEt is reduced in both *bushy* shoots and NAA-incubated roots. Based on this correlation of the levels of IAA and its precursors, it is possible that the roots and shoots of pea operate in a similar manner in terms of IAA regulation.

The most important conclusion that can be drawn from the present research is that, in pea roots and shoots, the tryptamine and/or IPyA pathways to IAA are operative. The evidence presented herein clearly indicates that pathways through IAM, IAOx or IAN do not operate in pea roots. A tryptophan-independent pathway may be operating, but further examination

must be conducted to either confirm or refute this. Possible future approaches to determining the importance of each relevant pathway may include incubating plants with two substrates, and determining the isotope enrichment of IAA from each source, and isolating further genes coding for the steps involved. This research has helped to answer some of the most fundamental questions about auxin: How is it made? Where is it made? And how is its production regulated?

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